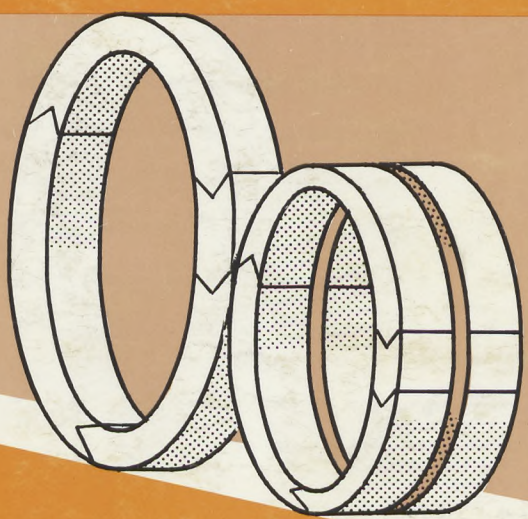
Effects of hyperthermia on mammalian cells



Guus van Dongen

EFFECTS OF HYPERTHERMIA ON MAMMALIAN CELLS

EFFECTEN VAN HYPERTHERMIE OP ZOOGDIER CELLEN

(Met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Abbreviations

Act.D	Actinomycin D
ATP	Adenosine-5'-triphosphate
CHI	Cycloheximide
Ci	Curie
cpm	counts per minute
DNA	Deoxyribonucleic acid
Do	time to reduce the survival in the exponential portion of the curve to 1/e
Dq	"quasi-threshold dose", defined as the intercept of the back-extrapolated straight line on the 100% survival axis
EDTA	Ethylenediamide tetraacetic acid
eIF	eukaryotic initiation factor
G1-phase	post-mitotic gap
G2-phase	pre-mitotic gap
GRP	Glucose regulated protein
Hepes	2-Hydroxyethylpiperazine-2-ethanesulfonic acid
hnRNP	heterogeneous nuclear ribonucleoprotein particle
HSP	Heat shock protein
HSTF	Heat shock transcription factor
kD	kilo-Dalton
M-phase	Mitosis
Mr	Molecular ratio
mRNA	messenger ribonucleic acid
MW	Molecular weight
NHCP	Non-histone chromosomal protein
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
S-phase	DNA synthesis phase
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminoethane

Chapter 1

General introduction

Mammalian cells, during their span of life, are exposed to temperature changes on which they response with more or less marked structural and functional adaptations. When such temperature change occurs rapidly, it is called a temperature shock. About 30 years ago, heat shock was recognized as a method for cell cycle synchronization. The effect of supra-optimal temperatures (hyperthermia) on physiological functions has gained renewed attention since data indicated that heat has beneficial effects against tumors. It was the start for studying the multiplicity of heat induced responses at molecular and cellular levels, the heat shock response. For clinical use of hyperthermia it is necessary that tumors can be forced to regress or to be sterilized while normal tissue is not damaged. In that respect it is of utmost importance to know the factors which influence cellular heat sensitivity.

An interesting aspect of heat stress physiology is the finding that a nonlethal heat treatment of mammalian cells can modify the subsequent response to elevated temperature. The increased thermoresistance induced by an initial heat treatment has been called thermotolerance. On the other hand, an initial heat treatment can also lead to heat sensitization. The molecular basis of thermotolerance is almost unknown although a number of data suggest a role for proteins whose synthesis is enhanced after heat shock, the so called heat shock proteins (HSP's).

Data presented in this thesis concern the heat induced effects on the proliferative capacity of synchronized cells and the modulation of these heat responses by changes in environmental conditions and pretreatment temperature. Moreover, the molecular mechanisms involved in getting over disturbance of cell proliferation caused by temperature shock are considered.

Why studying the effects of heat shock in synchronized cell populations? Tissue or tumor growth depends on growth in cell size and on the increase in the number of cells. Since increase in cell number is due to mitotic division of cells, the understanding of mechanisms that regulate cell division is desirable. The cell cycle is defined as the interval between two subsequent divisions and can be divided into four phases: post-mitotic gap (G1), DNA synthesis phase (S), pre-mitotic gap (G2) and mitosis (M). Knowledge of the cell cycle will give us a biological basis through which populations of cells grow in number, but even more important, will provide us with a framework to sort out the biochemical and molecular events relevant for cell division.

It may be clear that viability and proliferative capacity of mammalian cells can be affected by physical and chemical signals from their environment as well as by spatial restrictions. Heat is not a toxic agent only, but it can be considered as a physical signal which together with other physical (e.g. pH, osmolarity, oxygenation) and chemical signals (e.g. nutrients, growth factors) influence cell viability and cell proliferation. This may indicate that for an understanding of basic and specific processes of heat response the interaction between the cell and its environment must be considered. Furthermore, the interaction between the cell and its environment (e.g. serum factors and temperature) depends on the position of the cell in the cell cycle. Synchronized cells may be a very useful device for studying effects of heat on proliferation and for understanding of the mechanisms involved to encounter disturbance of cell proliferation caused by temperature shock.

Outline of this thesis

Current knowledge of the effect of hyperthermia on the biology of eukaryotic cells is reviewed in *chapter 2*. However it is impossible to cover all the different areas of hyperthermic research. Therefore most data concern effects of hyperthermia on eukaryotic cells in culture. This means

that data from e.g. clinical studies, in vivo studies and studies on hyperthermic treatment in combination with other treatment modalities such as irradiation and drugs are not considered in this thesis.

Chapter 3 deals with the capability of mammalian cells to withstand sudden changes in temperature. Surviving response of asynchronous and synchronized mouse neuroblastoma cells after hypo- or hyperthermic treatment was found to depend on cell cycle phase and on incubation time and temperature. Moreover those experiments show that hypothermia sensitizes cells to hyperthermia, also in a cell cycle dependent way. The capacity to proliferate was determined by evaluating colony forming ability.

Disturbance of cell cycling by a nonlethal heat treatment is described in *chapter 4*. Mouse neuroblastoma cells react to heat by inhibition of DNA- and protein synthesis and induction of cell cycle progression delay. Induction of mitotic delay depends on the cycle phase at the moment of heat application. Moreover it is demonstrated that cell division becomes thermotolerant after heat treatment. A relationship between reduction of protein synthesis and induction of mitotic delay has been considered.

Chapter 5 gives some evidence that elevation of temperature influences specific signals that regulate normal proliferation. It is demonstrated in this chapter that neuroblastoma cells depend on serum or growth factors for continuous growth. Serum protects cell cycle progression, protein synthesis and survival against heat in a cell cycle dependent fashion. The mechanisms involved in serum protection of cell cycle progression and cell survival seem to be different.

In chapter 3 en 5 it has been demonstrated that heat sensitivity can be modified by pretreatment temperature or by serum presence during heat treatment. In chapter 4 it is demonstrated that after heat shock the heat sensitivity of a cell alters. What is the molecular basis for regulation of heat sensitivity after varying environmental conditions? Results presented in *chapter 6* give evidence for a role of HSP's in proliferation after heat treatment. This conclusion was drawn from experiments in which cell cycle progression into mitosis and cell survival after heat treatment had been studied under conditions that enhancement of HSP synthesis after heat treatment was suppressed by actinomycin D. The synergistic effect of actinomycin D and heat on induction of mitotic delay and on induction of cell killing depended on cell cycle phase.

Heat sensitivity was modulated during cell cycle (chapter 3), by environmental signals such as serum (chapter 5) or by heat shock (chapter 4).

The question is raised whether common molecular mechanisms are underlying these modifications in heat sensitivity. *Chapter 7* shows the increase of thermoresistance of resting Reuber H35 hepatoma cells after growth stimulation with serum containing medium. The molecular basis for increase of thermoresistance upon growth stimulation is considered and a comparison is made with induction of acquired thermotolerance as has been observed in studies on fractionated hyperthermia. A role for non-histone chromosomal proteins (NHCP's) and HSP's in regulation of thermosensitivity both after stimulation with serum containing medium and after heat shock has been suggested.

In *chapter 8* the regulation of heat sensitivity of H35 hepatoma cells after heat shock has been further investigated. Heat induced alterations in NHCP's as well as in the synthesis of HSP's and their cellular distribution are described and related to the expression of thermotolerance.

Finally, in *chapter 9* the results described in the various chapters are summarized and discussed.

Chapter 2

Literature review

I. HEAT INDUCED CELL KILLING

Intrinsic heat sensitivity of mammalian cells

Kinetic analyses of heat induced cell killing are used by numerous investigators in order to relate cell killing with either general or specific molecular events. The motive for most of these studies is to find the biological basis of hyperthermia in cancer therapy. To compare intrinsic heat sensitivity of cells it is a prerequisite to use standardized culture conditions. Several studies indicate that the intrinsic thermal sensitivity of cells varies among cell lines. There is no evidence that cancer cells in general are more thermosensitive as compared to their normal counterparts (1-4).

A determinant in heat sensitivity of a cell is its position in the cell cycle at the moment of heat application. Experiments on synchronized cells demonstrated that cells in S phase were most sensitive, while cells in G1 phase were most resistant (5,6).

Modification of heat sensitivity by environmental factors

Heat sensitivity of cells is influenced by a number of physiological parameters in cell's environment such as pH, oxygenation, nutritional status and growth factors. It has been generally accepted that heat sensitivity of cells increases when pH during heat treatment is lowered (7-9). Oxygenation also may influence the sensitivity to heat but experimental data are far from uniform. A protective effect of acute hypoxia against heat killing of cultured cells was found by Bass et al. (10), a sensitizing effect by Harisiadis et al. (1) and Kim et al. (11), while several authors reported that heat sensitivity was not influenced by oxygen (12,13). Gerweck et al. (14) proposed that in studies where a sensitizing effect of hypoxia was observed also other environmental factors such as nutrients and/or pH might have been changed. They studied the effect of acute and chronic hypoxia under fully controlled nutritional and pH conditions and demonstrated that acute hypoxia did not alter heat sensitivity while chronic hypoxic conditions caused an increase of hyperthermic killing.

Another recognized factor in relation to heat sensitivity is the nutrient supply of cells. Hahn (12) demonstrated that heat sensitivity of Chinese hamster cells (HA_1) depends on presence of serum (or other components in the medium) before and during heat treatment. Some experimental data indicate which factors in serum containing medium may be important. It has been demonstrated that glucose starvation causes enhancement of cell killing (12,15). Also growth factors can alter heat sensitivity. Stimulation of quiescent Swiss mouse 3T3 cells with serum or with epidermal growth factor (EGF) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) markedly inhibited heat induced rounding-up of the cells (16). These data indicate that environmental conditions which are typical of tumors such as low oxygen pressure, insufficient nutrition and high acidity may increase the destructive effects of hyperthermia. Gerweck (17) therefore concluded that the rationale for hyperthermia in cancer therapy is accessory rather than intrinsic to the transformed state.

Effect of thermal history on heat sensitivity

The response of mammalian cells to elevated temperatures can be modified by hypo- and hyperthermic pretreatment. An interesting aspect of thermal biology is the phenomenon of acquired thermotolerance i.e. an

induced resistance to otherwise lethal heat shocks. When cells are exposed to a non-lethal heat treatment they acquire resistance to subsequent exposure at elevated temperatures.

Heat sensitivity of mammalian cells can be modified by a short exposure to elevated temperatures (heat shock) but also by adapting them to temperatures a few degrees above the usual growth temperature of 37°C. Li and Hahn (18) demonstrated that in parallel with the adaptation of Chinese hamster cells to 39°C and 41°, these cells became more heat resistant. Interestingly, Raaphorst et al. (2) demonstrated that heat sensitivity of mammalian cells, in general, could be related to the body temperature of the species from which the cell line was derived. Li and Hahn (19) generated a model for expression of thermotolerance. They propose that thermotolerance can be devided into three complementary and sometimes competing processes: a triggering event and events leading to development and decay of thermoresistance. Expression of thermotolerance can be modified by environmental factors such as nutritional conditions of the cell and the pH of the growth medium. Both, lack of nutrients (19) and low pH (20) inhibited the expression of thermotolerance.

A number of experimental data suggest that protein synthesis, more especially the by heat shock induced synthesis of a set of proteins (the so called heat shock proteins or HSP's) might play a role in thermoresistance (21,22). In studies on fractionated hyperthermia an intimate connection between formation of HSP's and development of thermotolerance was found (23-27). Also other inducers of HSP synthesis were active in induction of thermotolerance (28-30). Defective or lacking HSP synthesis in either a heat shock mutant of Dictyostelium (31) or after addition of cycloheximide (21-22) precludes the development of thermotolerance. Moreover, Laszlo and Li (32) selected thermoresistant cell lines of Chinese hamster fibroblasts which showed an elevated level of basal HSP 70 synthesis.

In contrast, thermotolerance in yeast does not require protein synthesis (33,34). The same conclusion was drawn from studies on growing pollen tubes (35) and Morris hepatoma cells (36). Boon-Niermeyer (37) proposed for three day old larvae of Lymnea stagnalis two states of thermotolerance after heat shock: an α- and β-state. The α-state is induced by temperatures between 31-38°C; it is responsible for an immediate response, independent of HSP synthesis and is rather unstable. The β-state is induced by temperatures $\geq$ 34°C, it shows a delayed response, depends on HSP synthesis and is stable.

Another interesting phenomenon in thermal biology is the ability of a high temperature pulse to sensitize cells to a subsequent exposure at lower heat shock temperatures (3,38). This type of experiments is referred to as "step down" heating. Step down heating has no effect on cells previously made thermotolerant (3).

Besides hyperthermic treatment also hypothermic preincubation alters the response to a subsequent heat shock. Sensitization to heat shock was observed in V79 cells grown at 33°C (39), HA-1 cells adapted to 32°C (18), Chinese hamster ovary cells adapted to 22°C and 30°C (40) and in Reuber H35 hepatoma cells (41). The degree of sensitization observed depended on the pretreatment temperature and the time during which cells were maintained at the conditioning temperature.

The underlying mechanisms for short-term and long-term alterations of heat sensitivity caused by alteration of pretreatment incubation temperature are generally unknown and further study is needed for a complete understanding of these experimental data.

II. HEAT INDUCED STRUCTURAL AND MOLECULAR ALTERATIONS IN EUKARYOTIC CELLS

Heat treatment of eukaryotic cells results in a multiplicity of molecular and cellular responses. Occurrence of these responses depends both on temperature and on the time of heat treatment (referred to as thermal dose). Knowledge of the heat induced alterations will give insight in the critical targets that might be responsible for cell cycle progression delay and cell death, but also in the cellular activities that protect the cell from irreversible injury and which allow recovery of normal cellular activities after heat shock.

The cell membrane has frequently been considered as an initial site and/or as a major target of heat action (42-44). A number of structural and functional changes have been described among which fluidization (45,46) and blebbing of the plasma-membrane and alteration of cell shape (16,47, 48,49). This injury may be reflected in alterations of membrane transport capacity as demonstrated for ions (50-54), amino acids (55), adriamycin (56), uridine (57) and polyamines (58). Heat sensitivity is connected with a low cholesterol-phospholipid ratio, indicating that heat sensitivity is related to membrane fluidity (59-61). Cholesterol is known to antagonize membrane fluidization (45). However Lepock et al. (62) did not find

evidence for a correlation between plasma-membrane fluidity and hyperthermic killing in V79 cells. They did not find lipid transitions in the range of 37°C - 60°C (46,63).

In eukaryotic cells a change in cell shape is accompanied by alterations in the organization of the cytoskeletal networks. Thomas et al. (49) reported flattening of HeLa cells and gerbil fibroma cells upon heat shock which was connected with the aggregation of vimentin around the nucleus. Essentially the same was found in some other cell types (64-66). In neuroblastoma cells, besides vimentin also microtubules became disorganized (67). Furthermore reorganisation of microtubules (68,69) and stress fibers (67) has been reported. Probably the responses of the cytoskeletons to heat shock depend on cell type. It has been shown that one or more of the major cytoskeletal networks, the actin-containing microfilaments, intermediate filaments composed of a number of different proteins depending on cell type and the tubulin containing microtubules, are essential for locomotion, cell shape, contraction and mitotic events, but also for movement of organelles and for uptake and secretory processes. Moreover cytosolic polysomes were found associated with the cytoskeleton. Therefore a relationship has been suggested between heat induced alterations in cytoskeletons and the quantitative and qualitative alterations in protein synthesis (see below). Recent data, however, indicate that a direct role of cytoskeletons in control of protein synthesis is improbable (66,67).

Heat induced changes in the cytoplasm also include the impairment of cell organels: disruption, fragmentation and hypertrophy of the Golgi complex (70); swelling of mitochondria and an alteration of the packing of the cristae (70); disruption of the lysosomal membranes (71) and damage to the endoplasmic reticulum (72). Damage to mitochondria may cause inhibition of respiration (73) which results in a diminished rate of ATP production and increased cell killing (15,74).

Also the translational machinery was altered: a basic protein of the ribosomal small subunit (ribosomal protein S6) became dephosphorylated (75,77). Moreover, initiationfactor eIF-2α was phosphorylated (78,79). eIF-2β became modified (79), eIF-4B was found dephosphorylated (79) while EIF-(3+4F) activity was inhibited without detectable change in structure of the factors (79,80). The alterations result in a reversible inhibition of protein synthesis which is connected with disaggregation of polysomes (47,81-83) and in a preferential translation of heat shock mRNA's relative to most preexisting mRNA's (84). Differential translation is

possibly caused by modulation of eIF-4F activity (80).

Structural and functional responses to heat have also been described for the cell nucleus. In parallel with rounding-up of Reuber H35 hepatoma cells, which seems to be linked with destruction of stress fibers (67), also their nuclei rounded up (85). It has been suggested that besides the cytoskeleton also the nuclear skeleton or nuclear matrix might be impaired during heat shock, but experimental proof is lacking. The nuclear matrix is involved in organization of interphase chromatin (86), is tightly associated with actively transcribed genes (87) and is implicated in hnRNA transcription, processing and transport. In fact, changes within chromatin have been observed in heated cells: Dewey et al. (88) suppose that protein within chromatin is the target for heat inactivation. Chromatin structuring appeared to be altered as determined by geometric and densitometric analysis of Feulgen stained nuclei (89) while in ultrathin sections no heterochromatin could be visualized (90). Furthermore hyperthermic treatment resulted in alterations in the in vitro phosphorylation of isolated non-histone chromosomal proteins (90). The amount of these proteins tightly associated with the nucleus increased upon heat shock (91,92). The amount of bound protein as well as cell killing increased with thermal dose. DNA synthesis was rapidly inhibited (93,94) while DNA strand breaks were observed with progressing incubation time after heating (95).

Hyperthermia also induced striking changes in the nucleolus: granular ribonucleoprotein (RNP) and intranucleolar chromatin disappeared. The material remaining in the morphologically homogeneous nucleolus was a large amount of closely packed fibrillar RNP (96). These structural changes are connected with changes in pre-rRNA synthesis. Inhibition of rRNA and thus ribosome synthesis is an early effect of heat shock. Pre-rRNA processing seems more heat sensitive as compared to transcription of rRNA genes (97). This leads to an accumulation of primary transcripts recognizable as fibrillar components of the nucleolus and an almost disappearance of the processing intermediates of pre-rRNA (the granular component of the nucleolus). Inhibition of nucleolar functions in CHO cells coincides with the appearance of new 95 kD phosphoproteins associated with nascent pre-rRNA (98,99).

Changes in the nucleus upon heat treatment are also connected with a reprogramming of mRNA synthesis. A reprogramming of transcription in Drosophila resulted in the activation of the genes encoding the HSP's and a concomitant decrease of transcription of non-heat shock genes (100-102).

Moreover a block of pre-mRNA processing and transport has been described (103,104). Mayrand and Pederson (107) found an incomplete assembly of hnRNA into hnRNP in Drosophila and HeLa cells and they propose that this leads to abortive processing of most mRNA precursors and favors the processing or export (or both) of others whose pathway of nuclear maturation is less dependent or even independent of normal hnRNP particle structures. According to this hypothesis the intron lacking HSP-mRNA's might belong to this last catagory. Probably, upon heat shock, reprogramming in favour of HSP synthesis takes place on at least three levels: transcription of genes, processing of mRNA precursors and translation of mRNA's.

As described before, heat can induce a state of decreased cellular heat sensitivity (thermotolerance). Cell death is probably due to disturbed cellular processes or structures, whereas induction of thermoresistance by hyperthermic treatment also appears to take place at very different cellular levels. A transient state of thermotolerance was demonstrated for cell morphology (47), cytoskeleton (105), protein synthesis (47,106) and DNA polymerase activity (107). Whether HSP's are involved in the protection of these structures and processes against heat, is totally unknown.

III. HEAT SHOCK PROTEINS

Introduction

In 1962, Ritossa (108) reported the alteration of puffing of salivary gland polytene chromosomes in heated Drosphila hydei larvae. This reflected a change in gene activity leading to synthesis of a specific set of proteins, the heat shock proteins (109). Now, the heat shock response, this is the induction of a small set of heat shock proteins, is known to be a property of most if not all organisms, both prokaryotic and eukaryotic (for reviews on heat shock response see: 110-117). Also other environmental stresses induce in most cell types such typical physiological reaction, although the spectrum of HSP's induced, varies for different stressors. Among the inducers of HSP's are oxidizing agents and drugs affecting energy metabolism, transition series metals, sulfhydryl reagents, chelating drugs, amino acid analogues, steroid hormones, ionophores, virus infection, ethanol, wounding and recovery from anoxia and glucose deprivation (for review see 115). HSP's are thought to protect the cell against a subsequent heat shock or stress condition. The precise intracellular

trigger for the response remains unknown, although it has been proposed that the general inducing principle is oxidation stress (118).

The pattern of induced proteins varies between species but a few major ones seem universal: All species so far examined have HSP's in the regions of 80-90 kD and 65-75 kD and sometimes one or more in the 20-30 kD region. Furthermore, analysis of cloned genes of Drosophila, Xenopus, yeast, mouse, Rainbow trout and E. coli, indicate that the HSP 84 and HSP 70 sequences have been highly conserved during evolution (119-123). The conservation of the gene products is revealed by the similar size, apparent isoelectric points and tryptic peptide patterns and by cross reactivity of polyclonal antibodies. Antibodies against HSP 70 and HSP 84 from chicken embryo fibroblasts cross-react with their counterparts from Drosophila, Xenopus, mice and humans (124). While there are striking similarities between a number of HSP's from different species it has been demonstrated that all types of HSP's may be modified e.g. HSP 84 was found phosphorylated (124,125), ADP-ribosylated (126) and methylated (127,128), while HSP 70 was found phosphorylated (129,130) or methylated (127,128). The functional significance and dynamics of these modifications are totally unknown.

Regulation of heat shock protein synthesis

As cited before, there is evidence for control of HSP synthesis at transcriptional and translational level and at the level of processing of mRNA precursors. Transcriptional controls have been observed in nearly all cell types. Exceptions are preblastula embryos (131,132) and Xenopus oocytes (133) which are loaded up with HSP-mRNA. The molecular mechanisms of transcriptional control is still not fully understood. Most experiments on regulation of heat shock protein synthesis concern HSP 70 in Drosophila. Studies to identify the molecular mechanism of transcriptional regulation can be divided into three categories: examination of DNA sequences responsible for gene activation, isolation of protein factors involved and analysis of the chromatin structure (134). This latter method includes detection of DNAse I hypersensitive sites which may be accessible to regulatory molecules (135). The mechanism of transcriptional activation is highly conserved during evolution because when cloned Drosophila genes are introduced into mouse cells, monkey cells or Xenopus oocytes they are only transcribed when the recipient cells are shocked (136,137). Deletion mutants were constructed and the DNA sequence responsible for regulation

located. Pelham and Bienz (138) found in such studies a promotor site 60-70 nucleotides upstream of the start of transcription. Comparison with the promotors of other Drosophila HSP genes revealed homologous sequences, referred to as Heat Shock Element (HSE). This region appeared to be DNAse I sensitive in control cells but insensitive in heated cells indicating that protein might have bound during heat shock. Recently, Parker and Topol (139) isolated a heat shock transcription factor (HSTF) with the expected properties: It is required for the in vitro transcription of cloned heat shock genes but has no effect on others. It has been suggested that HSTF promotes transcription by interacting with polymerase II (116). In fact, simultaneously with the changes in transcription patterns in Drosophila salivary glands redistribution of RNA polymerase II was observed. Also other authors have described protein factors isolated from cytoplasm or nucleus which are claimed to have a role in regulation of transcription (140-144). Bonner et al. (144) isolated a protein capable to induce heat shock puffs in isolated polytene nuclei which was totally different from HSTF. They suppose that HSTF probably needs to interact with this factor to become fully active. However, experiments to test such a model still have to be performed.

Another aspect of regulation of gene expression upon heat shock is the translational control. Exposure of eukaryotic cells to heat causes a decrease of protein synthesis rate. In mammalian cells an immediate cessation of initiation was observed followed by inhibition of elongation (81,83). Reduction of protein synthesis during heat shock of Drosophila cells seems to be due to concomitant reduction of initiation and elongation rates (145,146). Concomitant with the decrease of protein synthesis, HSP-mRNA's are preferentially translated. Control mRNA's of Drosophila and mammalian cells are sequestered and undertranslated, as has been demonstrated in cell-free translation systems (49,147-149). In contrast, the control mRNA's in yeast are degraded during heat shock (104,150). Ballinger and Pardue (151) suppose that the preferential translation of HSP-mRNA's might be useful as a response to withstand a heat shock because this allows the cell to concentrate merely on production of HSP's. Moreover, sequestering of control mRNA's might be of utmost importance for developing organisms which during oogenesis accumulate maternally encoded mRNA.

The underlying mechanism behind heat induced alteration of translational control is not understood. In heated Drosophila and HeLa cells

preferential synthesis of HSP's is due to translation selection of HSP-mRNA's from a pool of control mRNA's (84). It has been speculated that HSP-mRNA's might be preferentially translated because they contain certain structural peculiarities which allows the ribosomal apparatus to discriminate them from control mRNA's (152). However, this possibility seems unlikely because no competition was observed when control-mRNA's and HSP-mRNA's were translated together in a cell-free translational system made from reticulocytes or from wheat germ (101,148). By performing in vitro translation, Scott and Pardue (153) demonstrated that crude ribosomes isolated from heated cells were altered. Since no protein synthesis is needed for the induction of alterations in translational control by heat shock, it seems likely that preexisting components become modified. Therefore alterations in modification of ribosomal protein S6 and/or initiation factors as described on page 15 probably form the molecular bases for induction of heat shock translation control.

What are the mechanisms behind HSP synthesis repression? It has been demonstrated that the decline of HSP synthesis coincides with a recovery of normal protein synthesis (150,154,155). Results of DiDomenico et al. (154,155) indicate that synthesis of HSP 70 is autoregulated both at transcriptional and translational level: when synthesis of functional HSP 70 was blocked by addition of amino acid analoques or cycloheximide, HSP 70-mRNA synthesis continued much longer than normally. Moreover under these conditions the translation of control mRNA's was repressed for a longer time indicating that HSP 70 is involved in reestablishing the translation of normal cellular mRNA's. On the other hand, when actinomycin D was added at various time intervals after heat shock, translation of HSP 70 was extended the earlier transcription was stopped by act. D. The mechanisms of transcriptional and translational repression for HSP 70 are still unknown. Lindquist and DiDomenico (156) postulated that HSP 70 might destabilize its own messenger. In fact it has been demonstrated that HSP 70 is a mRNA binding protein (148,154). Moreover break down of HSP 70-mRNA seems to depend on the presence of functional HSP 70 (155). Whether HSP 70 also modulates the synthesis of other HSP's is not known yet.

Intracellular localization and possible functions of heat shock proteins

A number of facts suggests a role for HSP's in the structural and functional adaptation of cells to environmental stress conditions. Evidence

for this came from studies on fractionated heat treatment where the occurrence of thermotolerance i.e. an induced resistance to otherwise lethal heat shock, is connected with the presence of HSP's. Arguments in favour of this hypothesis are cited at page 13. Nevertheless, specific functions of HSP's are not known yet. An approach which has been used in the study for the functions of these proteins is to localize them within the cell by use of specific antibodies or by cell fractionation. HSP's are generally indicated by their apparent molecular weight in kilo-Dalton. However, as a result of variations between species and analytic procedures, a considerable heterogenity in molecular weight data exists for identification of a HSP. Therefore in reviews dealing on HSP's, collective terms are used for indicating their apparent molecular weights e.g. HSP 70 for the prominent eukaryotic HSP, whose M.W. is reported to be between 67 and 74 kD. The next intracellular locations and features of HSP's have been described:

HSP 110 is a mammalian stress protein which associates with nucleoli as has been demonstrated by use of rabbit antiserum (157). Treatment of cultured cells with deoxyribonuclease destroyed the organization of staining within the nucleus while ribonuclease released the antigen completely from the nucleus. Nuclear staining was diminished under heat shock conditions in transformed cells as compared to their normal counterparts (158). The protein is also present under control conditions.

HSP 100 (M.W. 92-102 kD) is a glycosylated stress protein which is also present under control conditions and which is located in the Golgi apparatus (159). This protein was found to be identical with the protein induced by glucose deprivation, virus infection or by exposure of cells to the calcium ionophore A23187 (159-163). This and another heat inducible protein of Mr 80.000 (159) are generally called the glucose regulated proteins or GRP's (160). Here they will be referred to as GRP's. The regulation of synthesis of these two proteins strongly depends on the cell line used, which is the reason that they sometimes are indicated as HSP's and sometimes as GRP's. Thus, these two proteins can be induced simultaneously with HSP's (159,164), but also separately or reciprocally from HSP's. Evidence for reciprocally related controls includes recent reports demonstrating induction of the HSP's and simultaneous deinduction of the GRP's during recovery from anaerobiosis or glucose starvation (165,166). Kasambalides and Lanks (167) demonstrated that glucocorticoids modulate the response of L929 cells to glucose deprivation by protecting the expected synthesis of Mr 80.000 and Mr 100.000 and causing the production

of HSP's. Also by pH and sulfhydrylreducing agents an induction of HSP's and GRP's was attained: The proteins of Mr 80.000 and Mr. 100.000 were induced in 2-mercaptoethanol treated cells and in cells exposed to low pH (168). HSP's were induced when cells were exposed to high external pH. These data give evidence for the idea that the redox state of sulfhydryls is involved in induction of GRP's and HSP's (see 118).

HSP 84 (M.W. 83-95 kD) was found in all species examined so far with an increased synthesis under stress conditions. The immunocytochemical data and cell fractionation studies show an exclusively cytoplasmic localization for this protein (124,159,169-171). HSP 84 was reported to be modified by phosphorylation (124,125), ADP-ribosylation (126) and methylation (127,128). The synthesis of HSP 84 can be modulated by glucose (165-167) and glucocorticoids. Glucose present in the culture medium inhibited HSP 84 synthesis. When additionally glucocorticoids (dexamethasone or hydrocortisone) were present the decrease of HSP 84 synthesis was not only reversed, but its rate of synthesis was induced to levels higher than normal. Insulin antagonized the effects of dexamethasone: it suppressed the synthesis of HSP 84 and prevented induction of the protein by heat shock (172). These data suggest that the change in HSP 84 synthesis induced by heat shock may reflect the operation of a normal physiological mechanism that regulates glucose metabolism. Furthermore, it appeared that high basal levels of this HSP may not necessarily protect cells against heat shock (171).

A more specific role of the 84 kD protein is indicated by studies on chicken embryo fibroblasts infected with Rous sarcoma virus. The transforming tyrosine kinase $pp60^{v\text{-}src}$ has been observed as a soluble complex with the 84 kD protein and another host cell protein of 50 kD (173-175). HSP 84 binds to newly synthesized $pp60^{v\text{-}src}$ and remains complexed with $pp60^{v\text{-}src}$ until the association of the $pp60^{v\text{-}src}$ protein with the plasma membrane takes place (176,177). While the purpose of this complex is not known, the 84 kD protein has been suggested to act in the transport or processing of $pp60^{v\text{-}src}$, or as a regulator of its enzymatic activity. Similar complexes with 84 kD and 50 kD have been observed with other retrovirus transforming proteins (175,178).

Recently another role has been suggested for this 84 kD protein. The protein was found to be complexed to avian receptors for progesterone, estrogens, androgens and glucocorticoids (179-184). At the moment there are no obvious functional relationships between steroid hormone action and

cellular stress responses or the activity of transforming proteins such as $pp60^{v-src}$. However with relation to steroid hormone action it has been suggested that the 84 kD protein could have a role in maintaining the receptor in an inactive form or in a receptor processing step that may occur following hormone stimulation (182).

HSP 68-70 (M.W. 65-74 kD). In most cells the major difference in the protein synthesis patterns between normal and stressed cells is the higher rate of synthesis of two proteins with apparent molecular wights of 68 and 70 kD. Partial proteolytic mapping of individual HSP 70 and the various isoforms of HSP 68 revealed distinct differences between the proteins in HeLa cells (158). However, in Drosophila, a multiplicity of coding genes for HSP 70 were shown to be 85% homologue with the single copy of HSP 68 (184). From HSP 68 as well as from HSP 70 isoforms were detected (186,187), some of them being phosphorylated (129,130) or methylated (127,128). Both proteins are present in cytoplasm of control cells however HSP 68 at lower level than HSP 70 (159). Following heat shock of mammalian and Drosophila cells there was an increase of HSP 68-70 levels in cytoplasm and in the nucleus. In the nucleus it was found in the nucleoli and other areas of condensed chromatin (158,169,188,189). During recovery period HSP's leave the nucleoli gradually. As described earlier, heat induced structural changes of nucleoli were connected with changes of pre-rRNA synthesis. Pelham (159) demonstrated that HSP 68 might accelerate the recovery of nucleolar morphology after heat shock which suggests a function for HSP 68 in reassembly of damaged rRNP's. HSP 68 and HSP 70 were also found attached to the nuclear matrix (158,170,190-192) and cytoskeleton (111,193,194) suggesting a structural function for these proteins. Furthermore, many laboratories have noted that at least HSP 70 is apparently associated with RNA (148,154,195), sometimes in ribonucleoprotein particles containing poly-A^+ RNA (196,197). Lindquist and DiDomenico (156) suppose that HSP 70 suppresses its own synthesis and the synthesis of other HSP's and releases the inhibition of preexisting mRNA translation.

One of the most interesting observations is the similarity between the DNA^{-k} gene from E. coli and the HSP 70 genes from eukaryotes (121). The DNA^{-k} gene product was shown to be essential for DNA replication in vitro and exhibits ATP-ase and autophosphorylation activities (198). Recently Wu et al. (199) demonstrated that transcription of the human HSP 70 gene is induced by serum stimulation and is linked to DNA synthesis.

The HSP 70 gene is also transcriptionally activated by two different oncogene products, adenovirus E1A (200-202) and mouse rearranged c-myc (203) and Wu et al. (199) therefore propose that HSP 70, in analogy with the DNA^{-k} gene product, might be essential for viral DNA replication. Furthermore, heat shock potentiated the stimulation by epidermal growth factor (EGF) and prostaglandin F2α (PGF2α) to initiate DNA synthesis (204,205). Potentiation of EGF-induced DNA synthesis and the induction of thermotolerance by heat shock showed a similar relationship to heat shock temperature and time, indicating that HSP's might be involved in the processes leading to DNA synthesis. All these data suggest that HSP 70 is essential for cell growth (probably for DNA replication). Moreover, it can be postulated that the transcriptional activation of the HSP 70 gene by viral transforming genes (200,201,206) and a cellular rearranged oncogene (203) might be an essential step in the molecular mechanism leading to a disruption of normal control of cell proliferation.

Small HSP's (M.W. 22-28 kD). Most studies on the small HSP's have been done on Drosophila. In Drosophila, following heat shock, at least four small proteins, HSP 22, HSP 23, HSP 26 and HSP 28 are induced. These proteins are synthesized in the cytoplasm and migrate to the nucleus and become associated with the nuclear matrix (169,179,190). Moreover they were found associated with RNA (196,207). All the small stress proteins are encoded within a single gene cluster (208) and were found to be related to each other and to the mammalian lens α-crystallin (209). The total set of small HSP's was induced by ecdysterone, the Drosophila molting hormone (210), while a subset was induced by some teratogens (211) indicating that the synthesis of the four small HSP's can be regulated separately. In mammalian cells phosphorylated isoforms of a 28 kD protein were described (212,213).

Heat shock protein synthesis under control conditions

Nowadays HSP's are defined as proteins whose synthesis is induced or enhanced under various conditions of cellular stress. In fact some of the HSP's are also synthesized under control conditions. For mammalian cells all HSP species are detected now in cells grown at 37°C and this knowledge is of considerable importance for elucidating their function. Heat shock gene expression was altered at well defined cellular growth stages. Expression of heat shock genes increased during embryonal development of Drosophila (214-216) and mouse (217,218), after addition of the molting

hormone ecdysterone to Drosophila cells (210), in mammalian cells after growth stimulation by serum (199) and by viral transformation (200,201, 206). Moreover, expression of HSP genes altered during the cell cycle (202) and decreased during cell differentiation (217,218). Elevated levels of HSP's were found in transformed cell lines as compared with their normal counterparts (201,219). These data suggest a role for HSP's (or some of them) in cell proliferation in general and in cell proliferation under stress conditions in particular.

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Chapter 3

Cell killing and sensitization to heat shock by hypothermic incubation of asynchronous and synchronized mouse neuroblastoma cells

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SUMMARY

The effect of hypothermia on cell survival and on subsequent response to hyperthermia was studied in asynchronous and synchronized Neuro-2A cells. Cell cycle progression was blocked at temperatures below 27°C. Immediately after shift to hypothermic temperatures, cells became more sensitive to hyperthermia. Development of thermosensitization was time and temperature dependent. Thermosensitization of cells by hypothermia was high at 0°C and between 15° and 30°C and less between 5° and 10°C. Sensitization started to occur before hypothermic cell death became manifest and developed gradually. Hypothermic cell death was observed when the cells were incubated for more than 1 day at temperatures between 0° and 24°C with a minimal cell death during incubation at 6°C. Thermosensitization of cells by hypothermia depended on the position of the cell in the cell cycle at the time of shift to hypothermic temperatures. Cells in late G_1 and early S phase became more thermosensitive than cells in G_1 or late S/G_2 phase. Furthermore G_1/S cells were more sensitive to prolonged hypothermia alone than G_1 or late S/G_2 cells. In contrast, late S/G_2 cells were most sensitive to hyperthermia alone. It is concluded that the temperature and cell cycle dependent way of hypothermic induced cell death was similar to the thermosensitization of cells by hypothermia. But thermosensitization became manifest prior to the actual cell death, following hypothermic treatment.

INTRODUCTION

Mammalian cells become rapidly inactivated following elevation of temperature above 41°-42°C. The hyperthermic response is, however, determined by a number of parameters, such as nutritional conditions (1), pH (2,3), incubation temperature before heat treatment (4-7) and the cell cycle phase of the cells (8).

In addition to supranormal temperatures (hyperthermia), a number of studies have been focused upon the effects of subnormal temperatures (hypothermia). These latter effects are of special interest since it is often necessary to store cells or organs at suboptimal temperatures, usually at either room temperature or at 4°C. Below 27°C, cell proliferation is arrested, and cells gradually lose their viability (hypothermic cell death) in a time- and temperature dependent way. Furthermore, the response to hypothermia is also determined by the phase of the cell cycle at the time of cold application (9).

The effect of hypothermic preincubation on cell killing induced by heat shock has been described in a few studies. Sensitization to heat shock was observed in V79 cells grown at 33°C (6), HA-1 cells adapted to 32°C (8) and CHO cells adapted to 22°C and 30°C (10). The degree of sensitization observed depended on the pretreatment conditioning temperature and the time during which cells are maintained at the conditioning temperature (10). Also mitotic CHO cells incubated at 1°-2°C for 4 hours became more sensitive to hyperthermia (4). Furthermore, hypothermic incubation influenced development and decay of thermotolerance in HA-1 cells (11). At 28°C development of thermotolerance was delayed while at temperatures below 32°C no decay of thermotolerance occurred. In a study on Reuber H35 hepatoma cells, effects of prolonged hypothermic preincubation on thermosensitization, radiosensitization and thermal radiosensitization were described (7). In these cells, a 24 h. hypothermic incubation caused maximal sensitization to hyperthermia and X-rays between 25°C and 30°C and no sensitization at 8.5°C. Development of thermotolerance at 37°C after an initial heating at 42.5°C was normal in hypothermic preincubated cells (24 h. at 25°C). Moreover, incubation of these hypothermic cells at 41°C resulted in a decreased expression of thermotolerance for heat or heat plus X-rays. A problem that has remained unsolved is whether the effect of hypothermia on thermosensitization is cell cycle dependent.

In this paper we describe the time and temperature dependence of hypothermia on cell killing and sensitization to heat shock of asynchronous and synchronized neuroblastoma cells.

MATERIALS AND METHODS

Cells and culture conditions

Mouse neuroblastoma cells were routinely grown at 37°C as subconfluent monolayer cultures in 150 cm^2 plastic Falcon flasks. The medium consisted of Dulbecco's modified Eagle's medium buffered with 25 mM Hepes pH 7.6 supplemented with 10% fetal calf serum (Seromed, Berlin). Synchronous cultures were obtained by selectively removing mitotic cells (mitotic indices of 95 percent) from asynchronous populations as described previously (12). The average cell cycle duration was about 14 h. and consisted of a G_1, S, G_2 and M period of 4, 6, 3 and 1 h., respectively, as analysed by pulse- and continuous tritiated thymidine incorporation followed by autoradiography and by determination of mitotic indices as described previously (12). Variation of cell cycle duration under these conditions, in different experiments, was less than 1 hour. At 1 hours after seeding 95% of the cells were in G_1, 5% in S phase. At 5 hours after seeding 40% of the cells were in G_1, 60% in S phase. At 11 hours after seeding 50% of the cells were in S, 42% in G_2 and 8% in M phase.

Survival determinations

In all experiments where cell proliferation was shown to be negligible, i.e. during hypothermic incubation at temperatures below 27°C, exponential growing cultures were trypsinized and single cells were seeded in appropriate numbers into 25 cm^2 flasks, and incubated 3 hours at 37°C before the experiment. In studies on cell cycle effects, synchronized cells obtained by mitotic shake-off were plated in appropriate numbers and temperature treatment started at various time intervals after seeding. In the Figures 1,2,5,6,7,8,9 and 10, cells were plated before temperature treatment.

In experiments where some cell cultures were incubated at temperatures above 27°C, cells were trypsinized and plated after hypothermic treatment then immediately followed by hyperthermic treatment (Figures 3 and 4).

At least in the temperature range between 0°C and 24°C, the survival after this procedure was similar to the survival of single cells plated 3 h. prior to cold incubation and subsequent heat treatment. Colony formation was judged 5 days later after fixation and Giemsa staining. Plating efficiencies ranged between 50 and 60%. In experiments on synchronized cells, survival curves are not corrected for cellular multiplicity. Standard errors

of the survival determinations are indicated by bars (unless smaller than symbol size of the figure).

Hypo- and hyperthermic treatment

For hypo- and hyperthermic treatments, culture flasks were immersed in a water bath. The heater was a circulating thermomix 1420 (Braun A.G. Melsungen, West Germany) provided with a proportional control of heating power giving stable temperature levels of ± 0.02 (S.E.) within the range used. Under these conditions temperature increased from 0° to 42°C in 8 min., from 25°C to 42°C in 6 min., within 5 min. from 37° to 42°C and from 24° to 45°C in 7 min.

RESULTS

Survival of asynchronous and synchronized Neuro-2A cells after hypothermic incubation

Multiplication of mammalian cells is restricted to a narrow temperature range. Neuro-2A cells gradually lost their ability to proliferate when incubation temperature was lowered in the range between 37° and 27°C. At 27°C the number of cells that entered the S phase or mitosis was reduced to less than 5% of the value at 37°C as determined in synchronized cells by respectively (a) [^{3}H]-thymidine incorporation followed by autoradiography, and (b) mitotic indices (data not shown). Based on these data the cell survival was determined as function of temperature in the range between 0° and 27°C.

The effect of hypothermia on survival of single cells obtained from asynchronous populations was determined during incubation at 0°, 6°, 10°, 14°, 19° or 24°C for various times ($\frac{1}{2}$ - 7 days) and subsequent incubation at 37°C for 5 days to determine their ability of colony formation. Survival of cells was a function of time and temperature of incubation (Fig. 1). Hypothermic induced cell killing was highest at 0° and 24°C. Lowering the temperature from 24° to 6°C resulted in an increase of cell survival while this capability decreased at 0°C. Cell survival curve parameters Do and Dq are listed in the legend of Fig. 1. A decrease of incubation temperature from 24° to 6°C resulted in increased Do and Dq values. Both values decreased again when cells were incubated at 0°C. At 24°C, the survival of Neuro-2A cells displayed a biphasic time dependency. After a shoulder and a log/lineair portion, by about 4 days the survival curve reaches a new, much shallower slope. Probably after this period of cold incubation, a

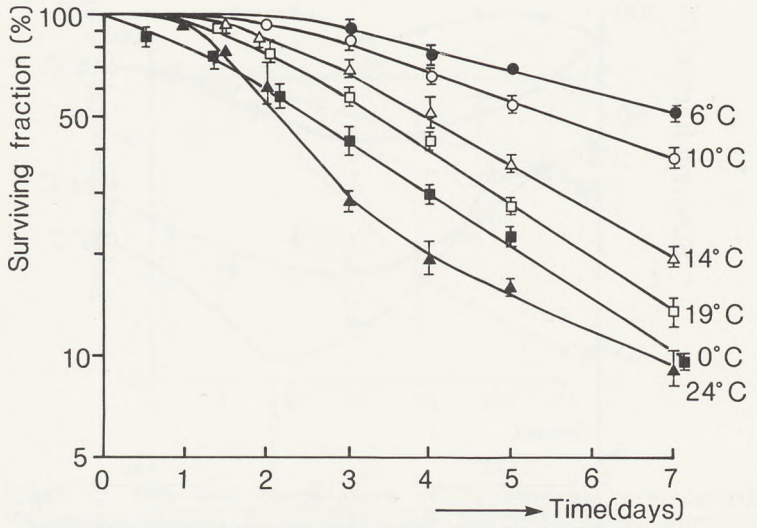

Fig. 1. Survival response of asynchronous Neuro-2A cells after exposure for various lengths of time to 0°, 6°, 10°, 14°, 19° or 24°C. Do and Dq values were calculated by least-squares analysis of the linear protion of the survival curves. Do values were 6.9; 15.8; 13; 7.9; 6.6 and 3.9 days, respectively, while Dq values were 0.2; 2.5; 1.8; 1.6; and 1.0 days, respectively. Do and Dq values of the 24°C curve are for the sensitive moiety. Data presented as means of 3 determinations; bars, S.E.

resistant cell population defines the shape of the survival curve.

The effect of hypothermia on the survival of synchronized cells is shown in Fig. 2. Cells were incubated at various times after mitosis for 4 days at temperatures of 0°, 6°, 18° and 24°C. Cells in G_1 and G_2 phase were less sensitive to hypothermic treatment as compared to S phase cells. In agreement with the data for asynchronous cells, killing of synchronized cells by hypothermia was maximal at 0° and 24°C and minimal at 6°C, irrespective of the cell cycle phase at the moment of cold application (Figures 1 and 2). However, small differences in hypothermic sensitivity appear to exist in the early G_1 phase of the cell cycle. Comparison of mid-G1 and mitotic cells shows an increased survival of mid-G_1 cells, at 0° or 6°C, and a decreased survival at 18°C or 24°C as compared to mitotic cells. But in order to prove the significance of the differences during this early cell cycle stage, more detailed study is required. These results clearly demonstrate that hypothermia induced cell killing is a cell cycle dependent phenomenon.

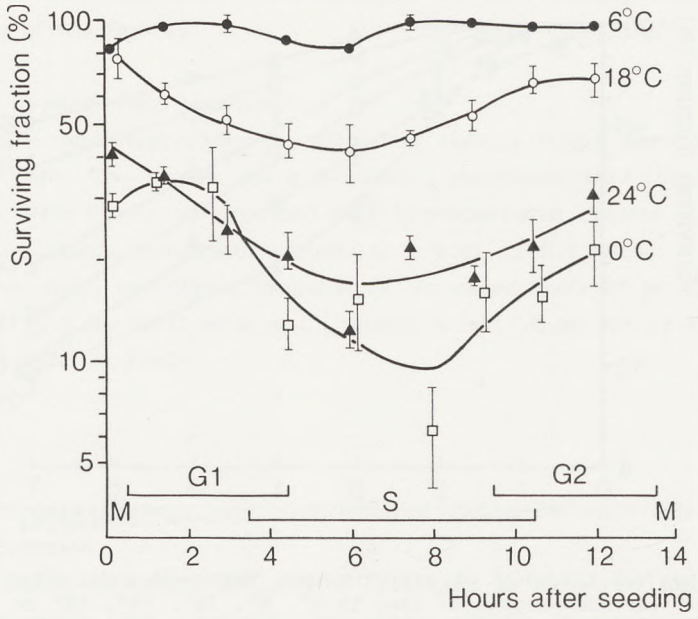

Fig. 2. Survival response of synchronized Neuro-2A cells after exposure to 0°, 6°, 18° or 24°C for 4 days as function of age in the cell cycle. The approximate durations of the fractions of the cell cycle are indicated at the foot of the graph. Data are presented as means of 3 determinations; bars, S.E.

Thermosensitization by hypothermia of asynchronous Neuro-2A cells.

Apart from the effect of hypothermia on its own we have studied the effect of a hypothermic preincubation of cells on hyperthermia induced cell death. The effect of duration and temperature of hypothermic preincubation on heat induced cell killing was studied in asynchronous cells by incubation at temperatures between 0° and 35°C for various time periods followed by a heat shock at 42.5°C for 1 hour. Because cell multiplication occurs at temperatures above 27°C during the temperature treatment, all cell cultures were trypsinized following the cold treatment. In the temperature range between 0° and 27°C in which no cell multiplication occurs, the cell survival using this procedure was similar to cell survival of cells plated 3 h. prior to cold incubation and subsequent heat treatment. Fig. 3 shows the effect of incubation at 4°, 19°, 31° or 37°C on heat shock induced loss of cell survival. After six hours of incubation at low temperatures (4°, 19° and 31°C, respectively) the cells became more

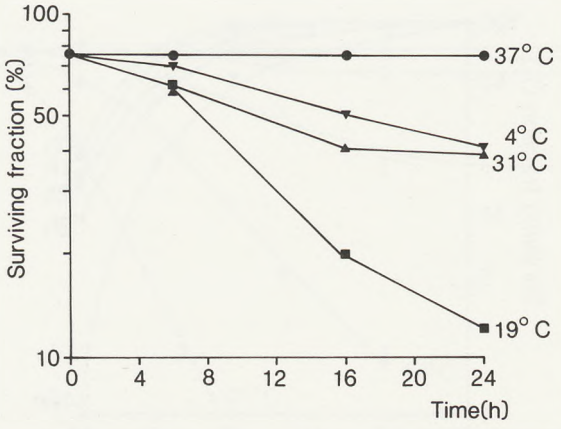

Fig. 3. Effect of time and temperature of hypothermic preincubation on thermosensitization of asynchronous Neuro-2A cells. Survival response of cells preincubated at 4°, 19°, 31° or 37°C for various lengths of time and subsequently heat treated at 42.5°C for 1 h.

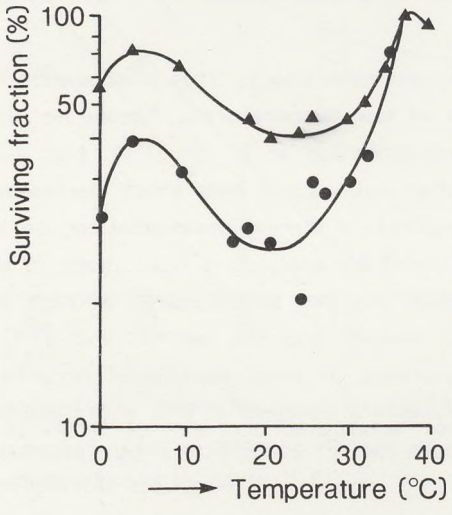

Fig. 4. Effect of temperature on thermosensitization by hypothermia of asynchronous Neuro-2A cells. Survival response of cells preincubated at various temperatures for 16 h. and subsequently heat treated at either 42°C (▲) or 43°C (●) for 1 h. Values were expressed as percentage of surviving fraction after hyperthermia without previous hypothermia. Values were corrected for hypothermic cell death.

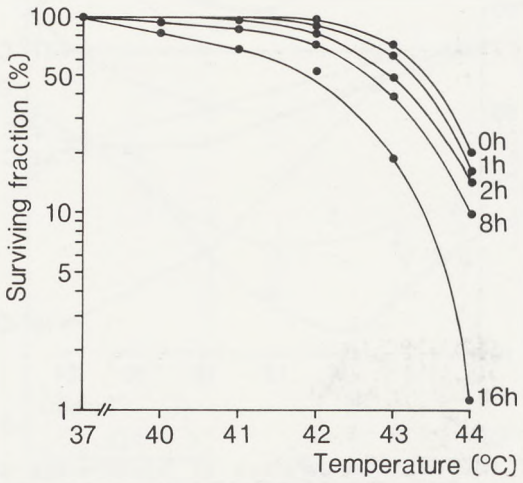

Fig. 5. Survival response of asynchronous hypothermic Neuro-2A cells after heat shock at various temperatures. Effect of hyperthermic treatment of 1h at 40°-44°C on survival of cells preincubated at 24°C for 0, 1, 2, 8 or 16 h. Data are presented as means of 3 determinations. Standard errors have been omitted but are less than 10%.

sensitive to a subsequent heat shock. This phenomenon is amplified during prolonged incubation at low temperatures. Almost no differences were observed between a preincubation at 4° or 31°C, but during incubation at 19°C, cell survival after subsequent heat shock decreased rapidly.

The effect of temperature during preincubation, on heat shock induced cell killing was performed by applying a heat shock of either 1 h. at 42°C or 1 h. at 43°C. When the two temperatures of heat shock were applied alone, the cell death was 0% and 50% for 42° and 43°C, respectively. As shown in Fig. 4, increase of heat sensitivity occurred at temperatures between 0° and 35°C, with a minimum at 6°C and a maximum at 24°C.

The effect of preincubation at 24°C on heat response has been studied in more detail by using various temperatures during heat shock. A heat treatment of 1 h. was applied at temperatures ranging between 40°C and 44°C. As shown in Fig. 5 survival of control cells decreased after 1 h. treatment at temperatures above 42°C. Upon prolonged incubation at 24°C cell death after 1 h. heat shock was initiated at lower heat shock temperature: after 8 h. and 16 h. low temperature preincubation, the initiation of cell death occurred at 41°C and 40°C heat shock temperatures, respectively.

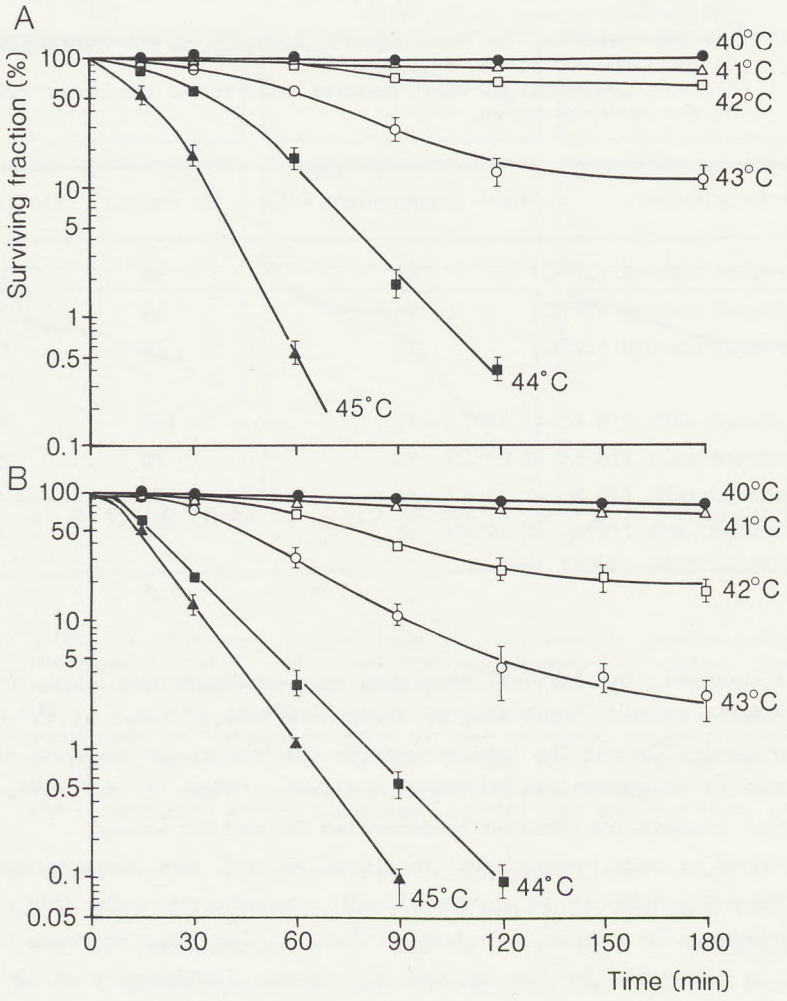

Fig. 6. Survival response of asynchronous Neuro-2A cells continuously heated at 40°-45°C for various lengths of time. A: control cells B: cells preincubated at 24°C for 16 h. Data are presented as means of 3 determinations; bars, S.E.

The modification of cellular sensitivity towards heat treatment with temperatures in the range of 40°-45°C was further established after a 16 h. incubation at 24°C (Fig. 6). Survival of asynchronously grown Neuro-2A cells (preincubated at 37°C), at temperatures above 40°C was dependent upon both temperature and time (Fig. 6A). In the range between 40° and 43°C the survival of Neuro-2A cells displays a biphasic time dependency.

TABLE 1. Parameters of survival curves of heat treated control or hypothermic preincubated Neuro-2A cells. The data were derived from the survival curves in fig. 6.
[a]Do was calculated by least-squares analysis of the linear portion of the survival curve.
[b]Dq was calculated by least-squares analysis of the linear portion of the survival curve.

Preincubation	Heat temperature (°C)	Do (min.)[a]	Dq (min.)[b]
Heat-treated control (37°C)	43	95	37
Heat-treated control (37°C)	44	39	27
Heat-treated control (37°C)	45	20	15
Preincubated cells (16 h. at 24°C)	42	139	34
Preincubated cells (16 h. at 24°C)	43	70	24
Preincubated cells (16 h. at 24°C)	44	38	5
Preincubated cells (16 h. at 24°C)	45	28	6

After a shoulder, the survival decreased in a log/linear way until, after 2 h. of heat treatment, cells display thermotolerance at least at 41°-42°C. The parameters Do and Dq which describe the loss of cell survival during incubation at increased temperatures are listed in Table 1. An increase of incubation temperature resulted in decreased Do and Dq values.

Survival of cells preincubated for 16 h. at 24°C was characterized by an increased sensitivity as compared with normothermic cells (Fig. 6B). The parameters Do and Dq are given in Table 1. The major increase in sensitivity is expressed by the reduced Dq values. Furthermore at 43°C, a contribution by a decreased Do value can not be excluded.

Thermosensitization of cells shifted to hypothermic conditions at different phases of the cell cycle.

As shown above, hypothermic treatment causes a loss of cell survival dependent upon the cell cycle phase of the cells (Fig. 2). In addition thermosensitization by hypothermia was studied with respect to its cell cycle dependency. Therefore, synchronized Neuro-2A cells were transferred from a temperature of 37°C to 0°, 6° or 24°C. Following the cold treatment,

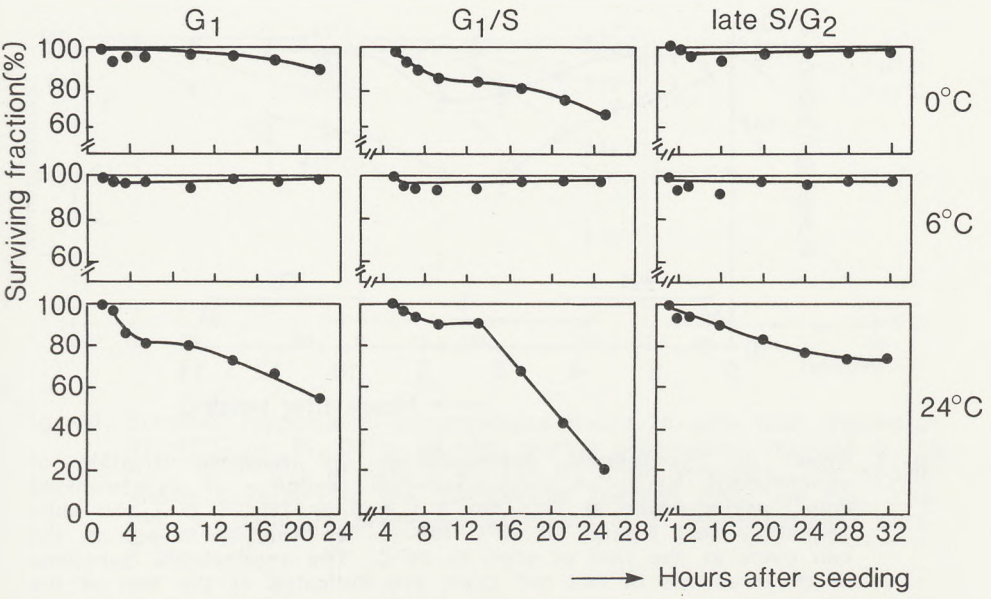

Fig. 7. Effect of time and temperature of hypothermic preincubation on thermosensitization of synchronized Neuro-2A cells. Survival response of synchronized cells shifted to 0°, 6° or 24°C at 2 h. (G_1), 5 h. (G_1/early S) or 11 h. (late S/G_2) after seeding and incubated for various lengths of time at these temperatures prior to heat treatment (1 h. 42°C). Values were corrected for hypothermic cell death. Data are presented as means of 3 determinations. Standard errors have been omitted but are less than 10%.

the thermosensitization was determined by applying a heat shock of 42°C. As shown in Fig. 7, G_1/S cells were more sensitized than late S/G_2 cells or G_1 cells by preincubation at 0° and 24°C. In Fig. 8, thermosensitization by 24°C preincubation of cells during their cell cycle is shown in more detail as compared to Fig. 7. Synchronized cells, at 1.5 hour intervals after seeding, were preincubated for 8 or 16h at 24°C and subsequently heat treated for 1h at 42°C. G_1/S phase cells were most sensitized to hyperthermia by hypothermic preincubation. Mitotic cells and early G_1 phase cells were sensitized to the lowest degree. These data show that hypothermic pretreatment alters the response of cells to hyperthermia in a cell cycle dependent way. Furthermore, these data show that the degree of cell killing after hyperthermic treatment of hypothermic cells is characterized by the temperature (Fig. 1 and 4) and cell cycle phase

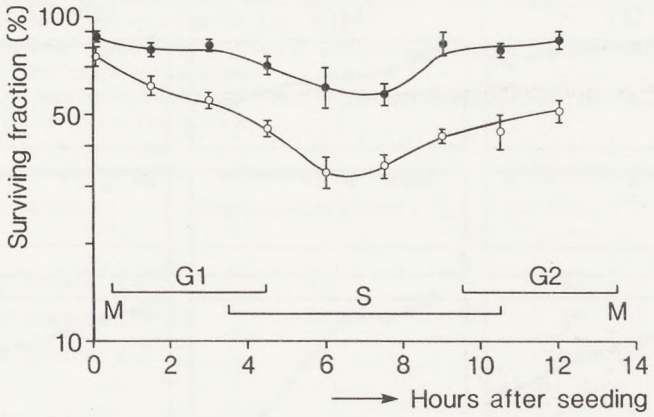

Fig. 8. Effect of hypothermic preincubation on thermosensitization of synchronous Neuro-2A cells. Survival response of synchronized cells, preincubated at 24°C for 8 (●) or 16h. (○) and subsequently heat treated at 42°C for 1h. as function of age in the cell cycle at the time of shift to 24°C. The approximate durations of the fractions of the cell cycle are indicated at the foot of the graph. Values were corrected for hypo- and hyperthermic cell killing. Data are presented as means of 3 determinations; bars, S.E.

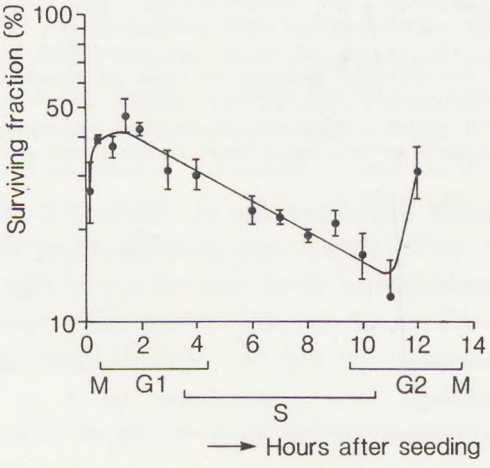

Fig. 9. Survival response of synchronized Neuro-2A cells heat treated at 44°C for 1h. at various time intervals after seeding. The approximate duration, of the fractions of the cell cycle are indicated at the foot of the graph. Data are presented as mean of 3 determinations; bars, S.E.

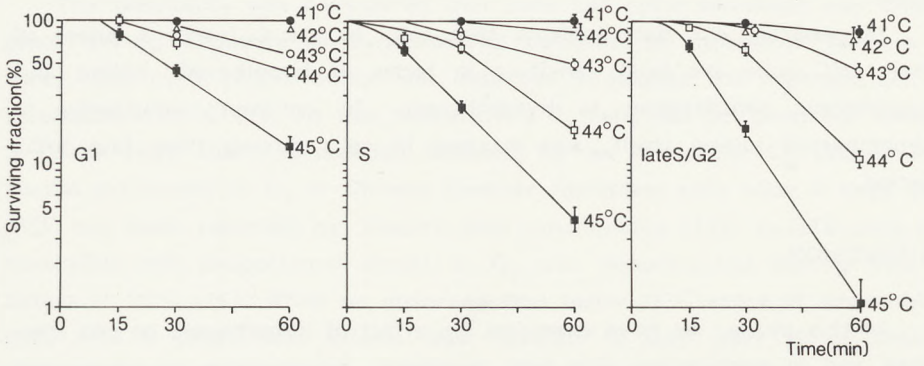

Fig. 10. Survival response of synchronized Neuro-2A cells heat treated at 41°-45°C for 15, 30 or 60 min. during progression through G_1 (2 h.), S (7 h.) or late S/G_2 (11 h. after seeding). For approximate durations of the fractions of the cell cycle see Fig. 2 or Fig. 8. Data are presented as means of 3 determinations: bars, S.E.

(Figures 2, 7 and 8). However, the data presented so far do not exclude the possibility that the cell cycle dependent pattern would be governed by hyperthermic sensitivity during the cell cycle. Therefore, survival of Neuro-2A cells exposed to hyperthermia was studied as a function of the position of the cell in the cell cycle at the time of heat shock. Fig. 9 shows the heat induced cell killing of cells which were heat treated for 1 h. at 44°C. After mitosis and early G_1, cells were relatively heat resistant, but this resistance decreased gradually during late G_1 and S phase, reaching a minimal value in G_2. Because of the loss of synchrony and the relatively short duration of G_2 and M phases of the cell cycle, it is difficult to correlate the maximal sensitivity with an exact localisation in G_2. However, 12 h. after mitosis, when most cells had entered G_2 and about 10% of the cells passed the second mitosis, survival increased again.

A detailed analysis of cell cycle dependency of the cell killing by a hyperthermic treatment is shown in Fig. 10. Cells were heat treated at 2, 7 or 11 h. after mitotic shake-off, i.e. during G_1, S and late S/G_2 phase. Irrespective of temperature of heat application in the range of 41-45°C, it appeared that G_1 cells were most resistant. Late S/G_2 cells were most sensitive towards the heat treatment. The Do values of the survival curves at 45°C were 58, 38 and 24 min. for G_1, S and late S/G_2 respectively and show a decrease during cell cycle progression. In contrast Dq values did not show clear differences for cells heated during those cell cycle stages.

The Dq values were 10, 8 and 11 min. for G_1, S and late S/G_2 cells respectively.

In general, our data indicate that cells in the G_1/early S phase of their cell cycle are most sensitive in terms of hypothermic killing and hypothermic sensitization to hyperthermia. In contrast, sensitivity to hyperthermic killing itself, was maximal in cells during their late S/G_2 period.

DISCUSSION

In this study, we have examined the effect of hypothermia on cell survival and in combination with heat treatment. Sensitization to subsequent hyperthermia developed gradually. After the shift to hypothermic temperatures, thermosensitization of cells by hypothermia was high at 0°C and between 15° and 30°C and less between 5° and 10°C. Sensitization to hyperthermia occurred before hypothermic cell death became manifest. It is conceivable that thermosensitization is related to hypothermic cell death because the temperature dependence of hypothermic cell killing and of thermosensitization are comparable. In both cases minimal effects were observed between 5° and 10°C. The general pattern of survival curves agrees with data of Nelson et al. (13) who found that survival of Chinese hamster lung cells (V79) after incubation at temperatures in the range 5°-25°C was maximal at 10°C.

The rate of thermosensitization was determined by the cell cycle phase at the moment of cold incubation. Hypothermic G_1 and G_2 cells were more resistant towards heat shock as compared to hypothermic G_1/S phase cell. Thus in synchronized cells also, thermosensitization appears to be related to hypothermic cell death since G_1 and G_2 phase cells were more resistant to prolonged hypothermic incubation than G_1/S cells (Fig. 2). In contrast, hyperthermic cell killing was highest for cells in the late S/G_2 stage of the cell cycle (Fig. 9).

Cold resistance of mitotic cells was relatively high especially at 18°C and 24°C (Fig. 2). Nagasawa and Dewey (14) reported that Chinese hamster mitotic cells are very sensitive to cold storage at 1°C. They demonstrated that metaphase cells are very sensitive to cold storage while doublet cells (anaphase-telophase) were cold resistant. The neuroblastoma mitotic cell population described here consisted of 25% single cells and 75% doublets. The great fraction of doublets in the mitotic cell population may account for the relatively high resistance of neuroblastoma mitotic cells to

cold treatment.

The possibility was considered that some cell cycle movement may take place during exposure of synchronized cultures to suboptimal temperatures. Cell cycle redistribution was observed in Reuber H35 hepatoma cells incubated at 25°C (7). At this temperature G_1 cells did not progress while S phase cells slowly proceeded followed by an arrest in G_2 phase. A partial synchrony in G_1 of Chinese hamster fibroblast cells after 4 days at 25°C has been reported by Shapiro and Lubennikova (15). In 3T6 cells a reversible and proportional arrest in G_2 was demonstrated during incubation at 15°C (16). While we observed that below 27°C entry of Neuro-2A cells into S phase and mitosis was reduced to less than 5% of their respective values at 37° (data not shown), we can not exclude that cells accumulated in G_2. If this accumulation occurs, this partial synchronization might be responsible for the observed thermosensitization of the cell population. However, hardly any cell death occurred after 1 hour treatment at 42°C irrespective the cell cycle phase, while about 40% of cells preincubated at 24°C for 16 h. was killed by such a heat treatment. Furthermore, thermosensitization occurred in the whole temperature range between 0° and 24°C with almost the same cell cycle dependency making it unlikely that synchronization effects are responsible for sensitization. Especially between 0° and 10°C the metabolic activity is (probably) too low for cell cycle progression. Moreover, we studied survival of synchronized cells incubated at 24°C for 1-7 days. The shape of the survival curves was the same as in Fig. 2 and was independent of time of incubation at 24°C (data not shown).It thus seems appropriate to conclude that thermosensitization by hypothermia is not caused by any cell cycle redistribution and that another temperature controlled mechanism must be involved.

A primary target for hypothermic killing and hypothermic sensitization is still unknown. It is also questionable whether the same or different targets are involved in the two processes. There are a number of data suggesting that phase transitions in the lipid bilayer of cellular membranes (from liquid to a more solid state) may be an important factor in hypothermic cell killing. Kruuv et al. (17) demonstrated in V79 cells the existance of lipid transitions in mitochondrial membranes but not in plasma membranes. Transitions were found at ca. 8°C and ca. 30°-32°C. They suggest that changes in the rate of cell inactivation and growth as function of temperature (7°- 10° and 30°C respectively) may be related to membrane lipid phase changes. Rule et al. (18) described that the lipid perturbers adamantane and 2,4- ditertiary butyl 5-hydroxytoluene (BHT) increased the survival of V79 cells at 5° but not at 20°C, indicating that

there may be a relationship between membrane viscosity and cold induced damage at 5°C but not at 20°C. Experiments with synchronized cells indicate that BHT protected cells against the effect of 5°C in all phases of the cell cycle, but protection was greatest in G_1 and early S phase (18). No evidence was found for a correlation between membrane fluidity and hyperthermic killing in V79 cells (19). Lepock et al. did not find lipid transitions in the range 37°-60°C (20,21). They suggest that while hypothermic killing commences with the onset of lipid transitions, hyperthermic killing commences at temperatures causing structural changes of membrane proteins. Although targets for hypothermic and hyperthermic cell killing may be different, our results indicate that interactions can exist between mechanisms for hypothermic and hyperthermic cell killing as demonstrated by heat treatment of hypothermic cells.

In conclusion, our results show that hypothermia sensitizes cells to hyperthermia. The rate of thermosensitization depended on (a) hypothermic temperature (b) length of hypothermic preincubation (c) hyperthermic treatment and (d) cell cycle phase at the moment of shift to hypothermic temperature. These data may have potential significance for optimal use of hyperthermia with or without radiotherapy in the treatment of cancer.

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Chapter 4

Comparative studies on the heat-induced thermotolerance of protein synthesis and cell division in synchronized mouse neuroblastoma cells

In: Int. J. Radiat. Biol. 46, 759-769 (1984)

G. van Dongen, L. van de Zande, D. Schamhart and R. van Wijk

SUMMARY

Mouse neuroblastoma (N_2A) cells react to a heat treatment by inhibition of DNA and protein synthesis and induction of cell cycle progression delay. Mitotic delay of heat-treated G1 cells correlates with reduction of protein synthesis and is due to an extensive delay of entrance into S phase, while the G2 phase of these cells is shortened. Mitotic delay of heat-treated G2 cells is more than in G1 cells and no correlation with protein synthesis reduction is found. In heat-treated G1 phase cells, both protein synthesis and cell cycle progression become thermotolerant to a second incubation at increased temperature. Moreover, the process of DNA synthesis becomes thermotolerant. In contrast, when heat-treated G1 phase cells have progressed into G2 phase and are then incubated at increased temperature, this G2 phase delay is not diminished. Apparently, additional targets for hyperthermia are present in late S and G2 phase cells.

INTRODUCTION

The use of hyperthermia as a method of treating cancer, either alone or in combination with radiation therapy or chemotherapy, has received increased attention in recent years (1-3). For a more rational use of hyperthermia as cancer therapy it is necessary to investigate the molecular mechanisms by which hyperthermia kills cells and causes delay in cell cycle progression. Earlier studies indicate a cell cycle dependent effect of hyperthermia on killing and progression through the cell cycle (4-6). Protein synthesis is of particular interest because for progression through the cycle, cellular protein synthesis is necessary (7-10) and protein synthesis is decreased after exposure of cells to elevated temperatures (11-13). We performed an analysis of thermotolerance of protein synthesis and cell cycle progression and this paper reports on their relationship in distinct phases of the cell cycle.

MATERIALS AND METHODS

Cell culture

Mouse neuroblastoma N_2A cells were routinely grown at 37°C as subconfluent monolayer cultures in 150 cm^2 plastic Falcon flasks. The medium consisted of Dulbecco's modified Eagle's medium with 25 mM Hepes (pH 7.6) supplemented with 10 per cent foetal calf serum. Synchronous cultures were obtained by performing a mitotic shake-off of a subconfluent monolayer. In this procedure 20 shakes (2 shakes/s) with fresh medium were used to detach mitotic cells. The population of cells detached was approximately 6 per cent of the total cell population (and 70 per cent of the number of mitotic cells present) and consisted of 95 per cent cells in mitosis, i.e. metaphase or double ball (telophase) stage. Subconfluent monolayer cell cultures underwent a shake procedure only once. These cells were plated at a density of 4×10^3 cells/cm^2. The average cell cycle duration was 14 h and consisted of a 4 h G1, 6 h S, 3 h G2 and 1 h M period.

Cell cycle duration

Progression of synchronized cells into the second cell cycle was assayed by counting 250 cells at hourly intervals in five to seven randomly chosen fields under a phase contrast microscope and determination of the number of mitotic cells in a double-ball stage. The standard deviation in the counting of the individual microscope fields was not greater than 8 per

cent. In order to use this method for establishing the effect of hyperthermia on cycle progression, the duration of double-ball stage was determined for untreated and previously heat-treated cells. Time lapse cinematographic studies showed an average duration of 21 ± 3 min and 24 ± 3 min for anaphase and telophase, of control and previously heat-treated cells, respectively. The method of counting described here, i.e. anaphase/telophase (double-ball) cells, has been applied because it is difficult to distinguish, after heat treatment, the few interphase cells detached from the substrate from cells in the pro-metaphase stage of mitosis. The arbitrary values, used in the figures, of the frequency of cells which have entered the second cell cycle, are cumulations of the hourly determined frequencies of double-ball cells.

Heat treatment

For heat treatment the culture flasks were immersed in a water bath. The heater was a circulating thermomix 1420 (Braun AG, Melsungen, F.R.G.) provided with a proportional control of heating power giving stable temperature levels of ± 0.02°C within the range used.

Protein and DNA synthesis

The rate of DNA synthesis was determined by [^{3}H]-thymidine incorporation. After labeling for 30 min with [^{3}H]-thymidine (1 μCi/ml medium; SA 50 Ci/mmol), the radioactivity was determined in the cold trichloroacetic acid-precipitable cell fraction. For autoradiography, the cells were washed twice with 1.1 per cent KCl and 1 mM EDTA, fixed with methanol: formaldehyde: acetic acid (85:10:5), dried with 70 per cent ethanol and covered with fine grain autoradiographic stripping plate AR10 (Kodak). After 1 week's exposure the radiographs were developed and the cells were Giemsa stained.

The rate of protein synthesis was determined by incubating cells in medium containing 2 μCi [^{3}H]-leucine per ml (SA 130 Ci/mmol) and measuring the radioactivity of the trichloroacetic-acid-precipitable fraction.

Chemicals

Foetal calf serum was obtained from Seromed. Radioactive chemicals were from the Radiochemical Centre (Amersham, England). All tissue culture components were from Gibco Grand Island Biological Co., Grand Island, N.Y. Cycloheximide was obtained from Boehringer Mannheim.

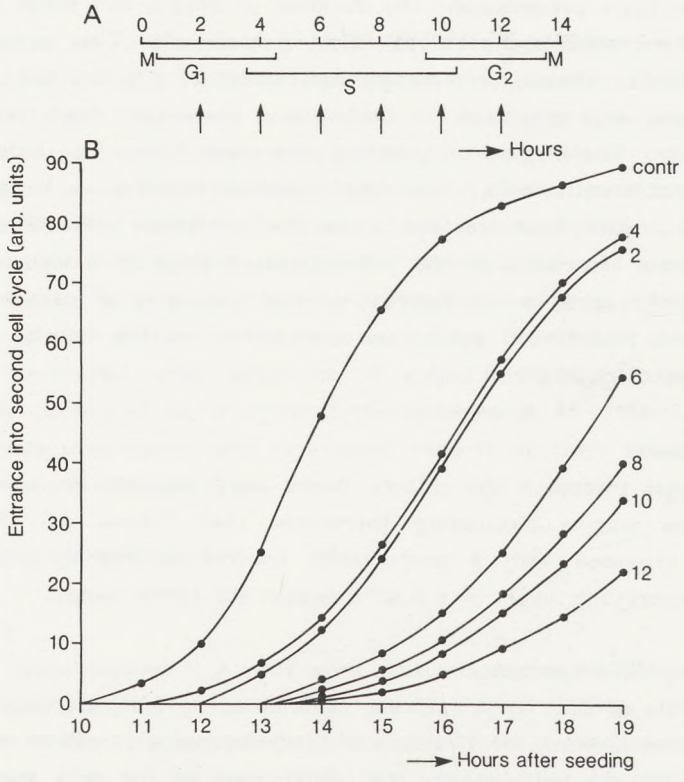

Fig. 1. Effect of hyperthermia on cell cycle progression of synchronized neuroblastoma cells. A, Cycle phases of cells synchronized by mitotic shake- off. Arrows indicate the time at which the cells were exposed to 42.7°C for 30 min. B, Entrance into second cell cycle following the heat treatment. Time of heat shock (h) after mitotic shake-off is indicated. Frequency of double-ball cells was determined in duplicate cultures. This experiment was repeated three times.

RESULTS

Effect of heat treatment on cell cycle progression of Neuro-2A cells

When a population of shaken mitotic Neuro-2A cells was incubated at 37°C more than 90 per cent of the cells regained their G1 appearance at 1 h after seeding. The cumulative values of mitotic indices assayed every hour showed an increase at 11 h and 50 per cent of cells had entered the second cycle at 13.6 h.

Analysis of the duration of cell cycle phases by continuous incorpo-

ration of [^{3}H]-thymidine and autoradiography showed that labelled nuclei became visible at 2 h and reached a 50 per cent value at 5 h after seeding. When mitotic cells were labelled with [^{3}H]-thymidine for 30 min (pulse labelling) at various time intervals after seeding, a similar increase of the percentage of labelled nuclei was found. A decrease of labelling index was found at 9 h after seeding as compared with continuous incorporation of [^{3}H]-thymidine. Subsequently a labelling index of 50 per cent following pulse label incorporation was found at 11 h. From these data the average durations of the different cell cycle phases were calculcated. Following a ½ to 1 h interval for the completion of the first mitosis after shake-off, the durations of G1, S and G2 were 4,6 and 3 h respectively.

When early G1 cells were incubated at 42.7°C for 30 min they entered the second cell cycle 2-3 hours later than control cells (figure 1). Cells treated during mid S phase were delayed about 5 h and treatment of late S and G2 cells caused a delay of more than 6 h. In order to discriminate between delay of progression and interphase cell death, cells in G1, S and G2 of their cycle were incubated at 42.7°C for 30 min and subsequently

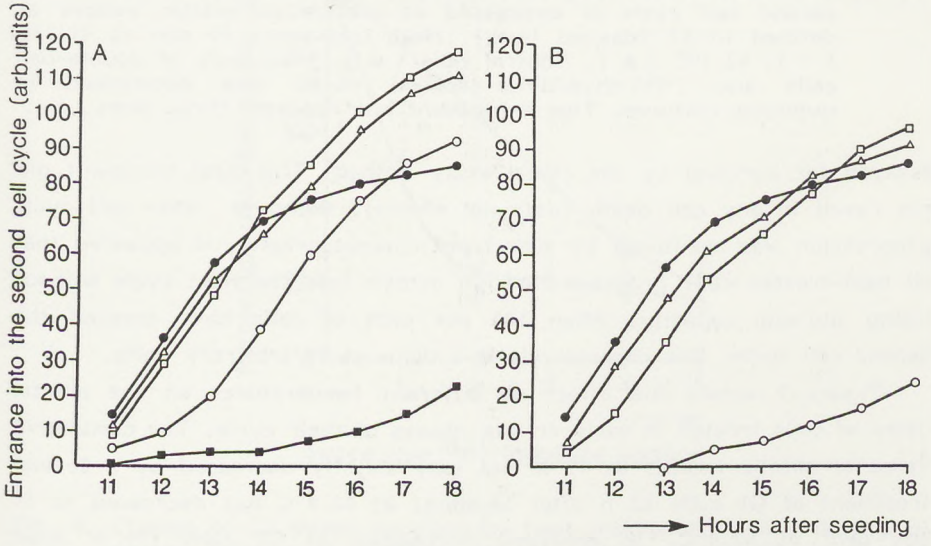

Fig. 2. Effect of temperature of heat treatment on cell cycle progression. Cells synchronized by mitotic shake-off were heat treated in G1 2 h after seeding (A), or heat treated in late S/G2 10 h after seeding (B). Heat treatment was for 30 min at 41.3 (△), 41.9 (□), 42.4 (○) or 42.9 (■). Control cells (●). Frequency of double-ball cells was determined in duplicate cultures. This experiment was repeated three times.

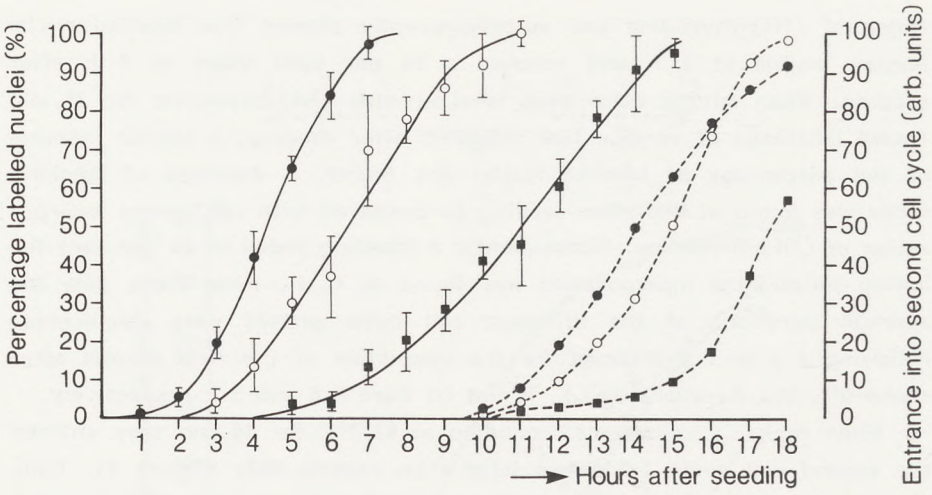

Fig. 3. Comparison of DNA initiation delay and mitotic delay, after heat treatment of G1 phase cells. Neuro-2A cells synchronized by mitotic shake-off were heat treated at 2 h after seeding. Entrance into S phase was assayed by continuous [^{3}H]-thymidine incorporation followed by autoradiography (continuous lines). Entrance into the second cell cycle is expressed as cumulative mitotic indices as defined in §2 (dashed lines). Heat treatment: 30 min at 41.7°C (○), 42.7°C (■). Control cells (●). Frequency of double-ball cells and [^{3}H]-thymidine labelled nuclei was determined in duplicate cultures. This experiment was repeated three times.

assayed for survival by the clonal assay method. This heat treatment did not result in any cell death (data not shown). Moreover, when cell cycle progression was monitored by time lapse cinematography, it appeared that all heat-treated cells progress through mitosis into the next cycle without losing division capacity. When 100 per cent of cells have entered the second cell cycle, this corresponds to a value of 90 arbitrary units.

Figure 2 shows the effect of different temperatures on the mitotic delay of cells treated in early or late phases of their cycle. The cumulative value of mitotic indices at 15 h was only slightly decreased by a 30 min treatment of G1 cells (2 h after seeding) at 42.4°C but decreased to 10 per cent at 42.9°C. In contrast, incubation of G2 cells (10 h after seeding) for 30 min at 42.4°C decreased the cumulative value of mitosis to 10 per cent. Apparently the difference in heat sensitivity of G1 and G2 cells is expressed by either a division delay of 4-5 h. or a temperature of 0.5°C (figures 1 and 2).

When cells are heat treated in early G1, progression through any of the

cell cycle phases (G1, S or G2) may be altered. When early G1 cells were incubated at either 41.7 or 42.7°C for 30 min and then reincubated at 37°C, entry into the S phase was delayed for 2.2 and 6.6 h respectively. In the same series of experiments entry into mitosis was only delayed by 1.0 and 3.6 h respectively (figure 3). Apparently the duration of the S + G2 phase was shortened.

The duration of the G2 phase of cells heat shocked in early G1 was assayed at the time when most cells were in late S/G2 phase of their cycle. [^{3}H]-Thymidine was added at 15 h after seeding and the percentage of labelled mitotic cells was determined subsequently at hourly intervals. There was a rapid decrease of unlabelled mitotic cells as a function of time and figure 4 demonstrates that 50 per cent of the G1-shocked cells had progressed from S to mitosis in less than 1 h. A control group of cells was

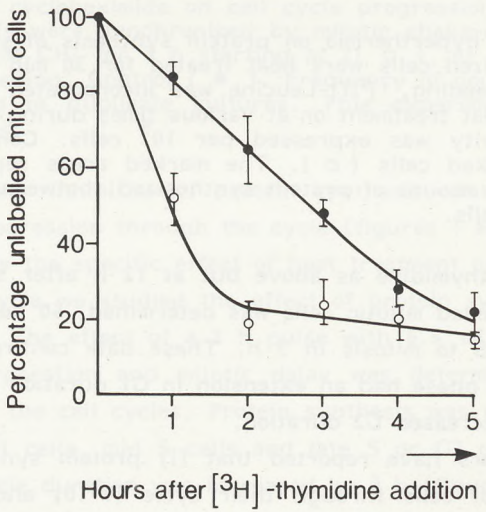

Fig. 4. Change of G2 phase duration by heat treatment of cells during G1 phase. Cells were synchronized by mitotic shake-off. In control cultures [^{3}H]-thymidine was added at 12 h after seeding (●). When cells were heat treated (30 min at 42.7°C) in G1 at 2 h after seeding, [^{3}H]-thymidine was added 15 h after seeding (○). After labelling started, mitotic cells were harvested by shake-off at hourly intervals and the percentage of unlabelled mitotic cells was determined by autoradiography. In duplicate cultures, 250 mitotic cells were counted per culture. The experiment was repeated three times.

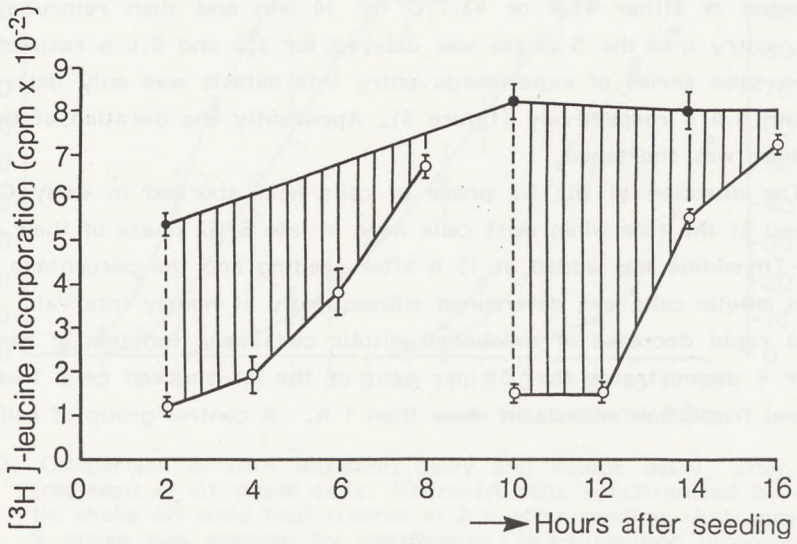

Fig. 5. Effect of hyperthermia on protein synthesis of synchronized cells. Synchronized cells were heat treated for 30 min at 42.7°C 2 or 10 h after seeding. [^{3}H]-Leucine was incorporated for 30 min either during heat treatment or at various times during recovery at 37°C. Radioactivity was expressed per 10^5 cells. Control cells (●), heat-shocked cells (o). The marked areas represent the differences in amount of protein synthesized, between control and heat-treated cells.

exposed to [^{3}H]-thymidine as above but at 12 h after seeding. When the percentage of labelled mitotic cells was determined, 50 per cent of the cells progressed from S to mitosis in 3 h. These data confirm that cells heat-treated in the G1 phase had an extension in G1 duration and a partial compensation by a decreased G2 duration.

Various authors have reported that (i) protein synthesis is essential for progression of cells through their cycle (7-10) and (ii) the rate of protein synthesis decreases during heat treatment (11-13). To determine the effect of heat treatment on protein synthesis of Neuro-2A cells, [^{3}H]-leucine incorporation of synchronized cells, heat treated during either their G1 phase or late S/G2 phase, was studied. When cells were incubated at 42.7°C for 30 min the rate of protein synthesis decreased during treatment to 20 per cent of control values (figure 5). Reincubation at 37°C resulted in recovery of protein synthesis in about 6 h. Apparently heat treatment of cells during the early or the late phases of the cell cycle

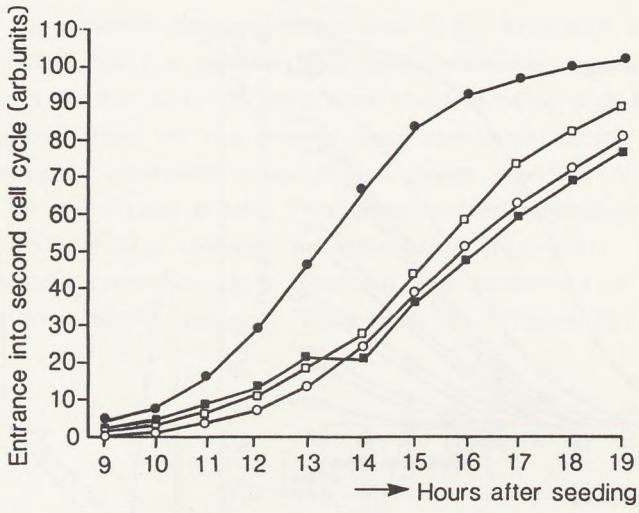

Fig. 6. Effect of cycloheximide on cell cycle progression of synchronized cells. Cells were synchronized by mitotic shake-off and incubated with 0.5 µM CHI for 3 h starting at 2(o), 6(□) or 10 (■) h after seeding. Control (●). Frequency of double-ball cells was determined in duplicate cultures. This experiment was repeated three times.

resulted in a similar inhibition of protein synthesis, but in a differential effect on their progression through the cycle (figures 1 and 5).

To demonstrate the specific effect of heat treatment on cells in the late part of the cell cycle we studied the effect of protein synthesis inhibition by cycloheximide. The effect of a 3 h pulse with 0.5 µM cycloheximide on [^{3}H]-leucine incorporation and mitotic delay was determined for cells in distinct phases of the cell cycles. Protein synthesis was reduced to 25 per cent for 3 h in G1 cells, mid S cells and late S or G2 cells. In all cases the median cell cycle duration was increased by 3 h (figure 6).

Combining the results described above it appears that the decrease of protein synthesis of G1 cells caused by heat treatment or cycloheximide pulse treatment is reflected in an increase of the duration of the median cell cycle time. In contrast, heat treatment of late S or G2 cells results in a much larger increase of the median cell cycle duration than was expected from the inhibition of protein synthesis alone.

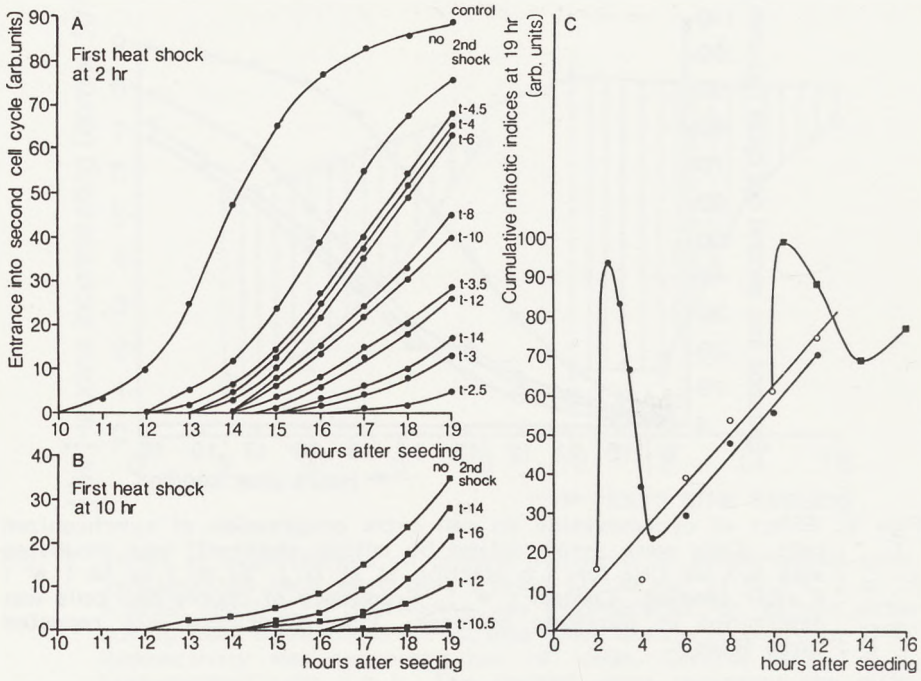

Fig. 7. Effect of fractional hyperthermia on cell cycle progression of synchronized neuroblastoma 2A cells. Synchronized Neuro 2A cells were heat shocked 42.7°C for 30 minutes at time intervals after seeding as indicated in the figure. Amount of double-ball cells was determined in duplicate cultures. This experiment was repeated three times.
A. First heat shock was at 2 h after seeding (●);
B. First heat shock was at 10 h after seeding (■);
C. Difference between the cumulative values of mitotic indices 19 h after seeding of control cells and cells shocked once or twice (42.7°C for 30 min) with different intervals at 37°C. In figure 7C the following symbols were used:
Cells heat-shocked twice at 2 and X hours after seeding (●);
Cells heat-shocked twice at 10 and X hours after seeding (■);
Data for cells heat-shocked once at X hours after seeding were derived from figure 1 (○).

Effect of a second heat shock

The effect of a second heat shock on cell cycle progression was studied in early G1 cells (figure 7A). Cells were heat treated (30 min, 42.7°C) at 2 h after seeding and subsequently reincubated at 37°C, and then exposed to a second heat treatment of 30 min at 42.7°C at distinct

time intervals. A single 30 min heat shock produced a median cell cycle time of 17 h and a mitotic delay of about 3 h. Continuous heat shock of 60 min resulted in a mitotic delay of more than 9 h. Also with intermediate incubation at 37°C for 1 h between two heat treatments the mitotic delay was more than 9 h. But when G1 cells were shocked twice with a 2 h interval at 37°C, the effect of the second heat shock on mitotic delay decreased. Prolonged intermediate incubation again resulted in increased mitotic delay by the second shock. This delay was approximately similar to the effect of a single heat shock at the later stage (figure 1).

When the first continuous 1 h treatment was performed in S/G2 cells this resulted in a mitotic delay of more than 9 h (figure 7B). But when

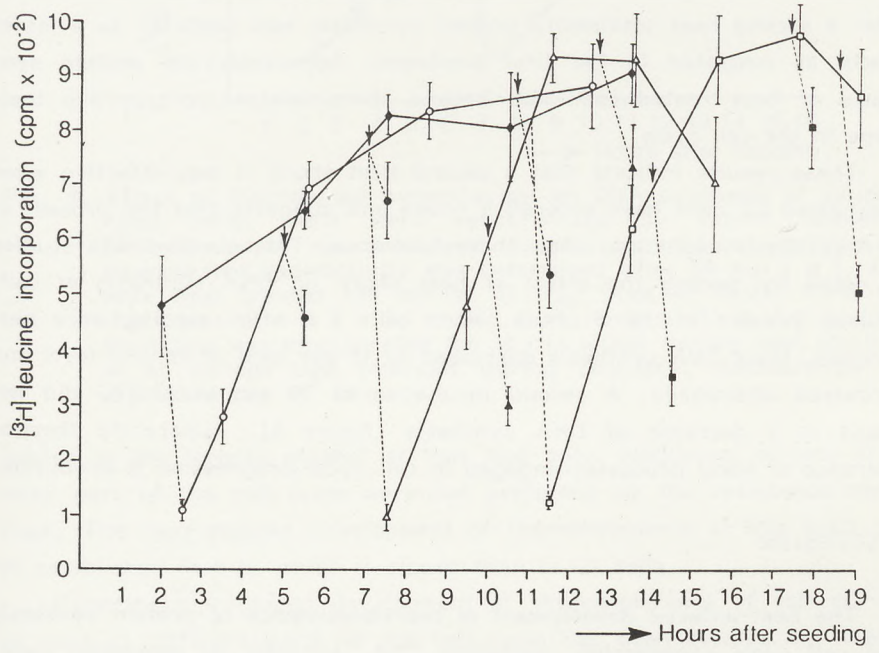

Fig. 8. Effect of fractional hyperthermia on protein synthesis of synchronized cells. Synchronized cells were heat treated (30 min at 42.7°C) once or twice at various time intervals after seeding. First heat shock was at 2 (○), 7 (△) or 11 h (□) after seeding. [^{3}H]-Leucine was incorporated for 30 min either during heat treatment or at various time intervals during recovery. Arrows indicate the time of a second shock of 30 min at 42.7°C. Incorporation of [^{3}H]-leucine during the second heat shock is presented by the closed corresponding symbols (●▲■). Control cells (♦). Radioactivity was expressed per 10^5 cells.

S/G2 cells were shocked twice for 30 min with an interval of 2-4 h the effect of the second heat shock on mitotic delay was decreased and again similar to the effect of a single heat shock at that stage. Apparently the heat-induced thermotolerance does not influence the effect of heat treatment at later stages of cell cycle progression (figure 7 C). The effect of a second heat shock on protein synthesis of preheated G1, mid S and late S/G2 cells is demonstrated in figure 8. Cells were preshocked (42.7°C, 30 min) at 2,7 or 11 h after seeding and subsequently allowed to recover for distinct time intervals at 37°C, followed by a second shock at 42.7°C for 30 min.

Immediately after the first shock, protein synthesis was decreased to 15 per cent of the control value. During the subsequent period at 37°C, after a second heat treatment, protein synthesis was inhibited to a lesser extent as compared to the first treatment. Apparently the protein synthesis of heat-treated cells had become thermotolerant irrespective their stage in the cell cycle.

These results indicate that a second heat shock is less effective when pre-heated G1 cells have entered S phase and suggests that the process of DNA synthesis might also show thermotolerance. This question was studied in detail by testing the effect of heat shock on DNA synthesis of cells actually present in the S phase. When cells 6 h after seeding were heat shocked, their DNA synthesis decreased to 15 per cent of control level and recovered afterwards. A second incubation of 30 min at 42.7°C did not result in a decrease of DNA synthesis (figure 9). Apparently thermotolerance of many processes engaged in cell cycle progression is produced.

DISCUSSION

The heat-induced development of thermoresistance of protein synthesis and cell cycle progression, including DNA synthesis in mammalian cells, has not previously been described, although recently thermotolerance of protein synthesis was found for hepatoma cells in culture (14). The progressive lengthening of mitotic delay when Neuro-2A cells were heat shocked in later phases of their cycle is generally consistent with delays following either continuous or short incubation at increased temperatures of other cell lines (6,15-20). This suggests extra targets for hyperthermia in the later part of the cell cycle.

The heat-induced thermotolerance of cell cycle progression of G1 cells is developed in 2 h, but the kinetics of development suggest a resistance

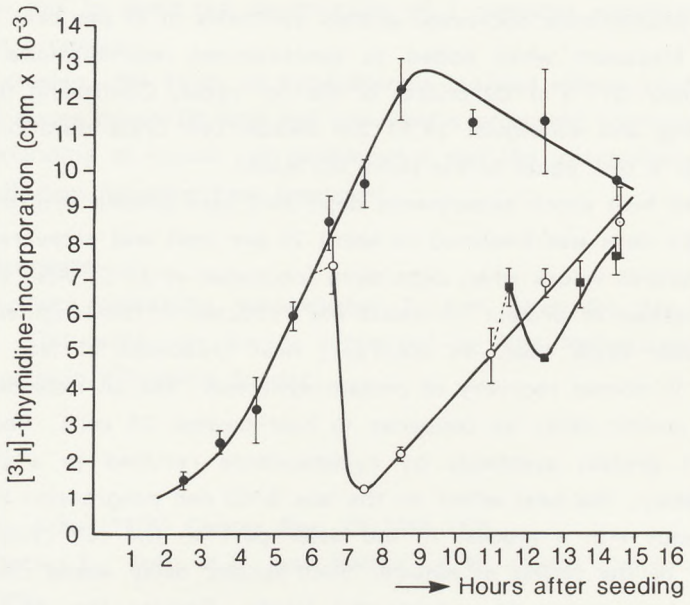

Fig. 9. Effect of fractionated hyperthermia on DNA synthesis of synchronized cells. Cells were synchronized by mitotic shake-off. [^{3}H]-Thymidine was added at various times after seeding and incorporated radioactivity was determined after 30 min (●). Cells were heat treated (30 min at 42.7°C) once or twice. First heat shock was at 6 h (○); second heat shock at 11 h (■). [^{3}H]-thymidine was incorporated for 30 min either during heat treatment or at various time intervals during recovery. Radioactivity was expressed per 10^5 cells.

limited to the targets present at that time only. Additional targets in the later part of the cell cycle were not protected by the resistance mechanism. The heat-induced development of thermotolerance of late S/G2 cells is established in 4 h, which is slower than in G1 cells.

Hyperthermia-induced development of thermotolerance of cell survival in cells at different parts of the cell cycle has been studied by Read et al. (20). They showed that development of thermotolerance for cell survival was more rapid in G1 than in G2. This paper describes the heat-induced development of thermotolerance of protein synthesis. Also in this case the thermotolerance of protein synthesis was developed more rapid in G1 than in G2 cells.

Heat-induced development of thermotolerance of cell cycle progression of G1 cells is probably due to resistance of protein synthesis. This causal relationship is suggested by different lines of evidence. Thus, pulses of

0.5 uM cycloheximide decreased protein synthesis to 25 per cent during the time of treatment when added to synchronized neuroblastoma N_2A cells during their G1, S or G2 phases of the cell cycle. Confirming the findings of Rønning and colleagues (8,9) the median cell cycle duration was prolonged by a time equal to the pulse duration.

In the heat shock experiments described here protein synthesis of heat-treated G1 cells was inhibited to about 20 per cent and recovered in about 6 h to control values when cells were incubated at 37°C. Also in this case the depression of protein synthesis was reflected in the lengthening of the median cell cycle time. In contrast, heat treatment of late S/G2 cells resulted in normal recovery of protein synthesis, but an extended lengthening of mitotic delay as compared to heat-treated G1 cells. Because inhibition of protein synthesis by cycloheximide resulted in a proportional mitotic delay, the heat effect on the late S/G2 cell progression indicates an interference with a process in the latter part on the cell cycle, which is involved in the timing of mitosis. Such mitotic delay would cause protein levels to accumulate to supranormal levels. Besides the development of thermotolerance of protein synthesis and cell cycle progression, several other cellular characteristics became resistant following hyperthermia. This paper shows the development of thermotolerance of DNA synthesis, which might be explained by the relationship between DNA and protein synthesis. Thus, inhibition of protein synthesis caused decreased DNA synthesis, and development of thermotolerance for protein synthesis can be expected to protect DNA synthesis also.

Thermal radiosensitivity is another characteristic which became resistant after hyperthermia of rat hepatoma cells (21). Moreover, the onset of cellular injury, included rounding-up of substrate-attached rat hepatoma cells, also becomes resistant to a second heat shock (14). In contrast ion transport was equally sensitive after hyperthermia and thermotolerance was not developed (22).

This paper demonstrates that heat treatment of G1 cells resulted in lengthening of G1 phase but the subsequent G2 period was shortened. Apparently, the signal for mitosis at that stage of the cell cycle is less sensitive to heat than the signal for initiation of DNA synthesis. This is in contrast to the situation of heat treatment of G2 cells, resulting in the appearance of tetraploid cells (20,23). Recently, we proposed a two-signal model for cell cycle control based on analysis of X-ray induced cell cycle perturbations in mouse osteosarcoma cells (24). It may be possible to give the differential hyperthermic effects a place in the model. However, an

explanation has to await the construction of a computer simulation model, which is in progress.

In conclusion, the study of hyperthermia-induced effects on the proliferation of mouse neuro-2A cells has provided a promising approach to both the understanding of normal cell proliferation and the interpretation of cell cycle distribution following heat treatment.

Acknowledgements

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Chapter 5

Effect of serum on heat response of synchronized mouse neuroblastoma cells: protection of cell cycle progression, protein synthesis and survival

In: Int. J. Radiat. Biol. (In press)

G. van Dongen and R. van Wijk

SUMMARY

The effect of serum and temperature elevation on proliferation has been studied in synchronized mouse neuroblastoma (Neuro-2A) cells. The effects of serum were studied on induction of 1) mitotic delay due to a non-lethal heat treatment (30 min. at 42.7°C) and 2) the loss of colony forming capacity after a more extensive heat treatment (45 min. at 44°C or a continuous 42.7°C heat treatment). The following results were obtained:

1) Under conditions of serum depletion, cell cycle extension of heated G1 phase cells was more than that of heated G2 phase cells. Serum protected against heat induced alterations of cell cycle progression in G1- but not in G2 phase cells. This effect of serum could be mimicked by a supplement of human transferrin, bovine pancreas insulin and selenium to the medium, and was correlated with protection of protein synthesis.
2) Serum also affected heat induced cell killing. Under conditions of serum depletion, G1 phase cells were more resistant to heat as compared to G2 cells. The presence of serum during heat treatment further increased the thermoresistance of G1 phase cells, but did not affect sensitivity of G2 phase cells. This effect of serum could not be mimicked by a supplement of transferrin, insulin and selenium.

These results indicate that serum protects G1 phase cells for heat induced changes of cell cycle progression as well as against heat induced cell death, but the mechanisms involved in both phenomena seem to be different.

INTRODUCTION

Elevation of temperature above 41°C resulted in a number of effects in cultured mammalian cells. Synthesis of both DNA (1,2) and proteins (2-5) were inhibited and cell cycle progression was delayed (2,6-12).

Heat treatment could ultimately result in the loss of division potential and cell death (10,13-16).

Some effects of heat shock were dependent upon the position of the cell in the cell cycle at the moment of heat application. Thus, in cell survival studies, G1 phase cells were found thermoresistant as compared to cells in late S phase (14,16). Moreover, mitotic delay following heat treatment was large in G2 phase cells as compared to G1 phase cells. (2,6,7,11,12). In contrast, heat induced reduction of protein synthesis was not cell cycle stage dependent (2).

Although effects of heat on cell cycle progression have been observed, it is not clear as yet whether the elevation of temperature influences specific signals that regulate normal proliferation. This paper deals with the effects of serum on heat induced alterations in cell cycling and survival of synchronized mouse neuroblastoma cells.

The presence of serum (or growth factors) is a major factor in the rate of cell replication of many mammalian cell lines in culture. Decrease of serum concentration results in inhibition of protein synthesis, growth arrest and accumulation of cells in their G1 phase. Serum deprivation at late cell cycle stages still inhibits protein synthesis, but could not abolish progression of cells to mitosis (17-23). These systems provide frameworks to study either the heat influence on serum dependent proliferation, or the influence of serum on heat induced delay of proliferation.

In this paper the importance of the presence of serum during heat treatment of cells in early and late cell cycle stages was established for protection to: 1) mitotic delay caused by a non-lethal heat treatment and 2) loss of colony forming capability after a more severe heat treatment.

MATERIALS AND METHODS

Cell culture

Mouse neuroblastoma (Neuro-2A) cells were grown in a 1 : 1 mixture of Dulbecco's modified Eagle's medium/Ham's medium (Gibco F12) buffered with 15 mM HEPES and 16 mM $NaHCO_3$, 7% CO_2, pH 7.5 (referred to as DFBH medium) at 37°C. For routine growth this medium was supplemented with 10% fetal calf serum (Sera-lab., Sussex, England). For growth in a defined serum-free medium, DFBH medium was supplemented with 100 ug human transferrin, 5 ug bovine pancreas insulin and 5.2 ng Na_2SeO_3 per ml (referred to as TIS).

Synchronous cells were obtained by selectively removing mitotic cells (mitotic indices of 95 per cent) from asynchronous populations as described previously (2). Cells were plated directly after harvest at a density of 4.10^3 cells/cm^2. The average cell cycle duration was about 9 h. and consisted of an average G1, S, G2 and M period of 3.5, 3.5, 1.5 and 0.5 h., respectively. Analysis of duration of the cell cycle phases was performed by pulse and continuous [^{3}H]-thymidine incorporation followed by autoradiography and by determination of mitotic indices as described previously (2).

In experiments in which serum-containing medium was replaced by serum-free medium, an intermediate washing procedure with phosphate buffered saline was applied.

Entrance into S phase

Entrance into S phase was assayed by continuous [^{3}H]-methyl-thymidine incorporation followed by autoradiography. Cells were incubated in medium containing 1 uCi [^{3}H]-methyl-thymidine (specific activity 86 Ci/mmol) per ml medium. Cold thymidine was added to the medium to a final concentration of 2 uM. For autoradiography, the cells were washed twice with 1.1% KCl and 1 mM EDTA, fixed with methanol: formaldehyde: acetic acid (85 : 10 : 15), dried with 70% ethanol and covered with fine grain autoradiographic stripping plate AR10 (Kodak). After exposure for 1 week the radiographs were developed and the cells were stained, using Giemsa stain.

Entrance into mitosis

Entrance of synchronized mitotic cells into their second cell cycle was determined microscopically by counting 250 cells at intervals of 1 hour in

5-7 randomly chosen microscopic fields of a phase contrast microscope and determination of the number of mitotic cells in a doublet stage. The standard deviation in the counting of individual microscope fields was not greater than 8%. This method of counting has been applied because it is difficult to distinguish, after heat treatment, the few detached interphase cells from cells in the pro- metaphase of mitosis. Furthermore, duration of anaphase and telophase was hardly influenced by heat treatment (2). The number of cells which had entered the second cell cycle was derived by accumulation of the hourly determined amount of doublet cells.

Protein synthesis

The rate of protein synthesis was determined by incorporation of L-(4,5)-^{3}H-leucine (specific activity 130 to 190 Ci/mmol). After labeling for 30 min. with [^{3}H]-leucine (2uCi/ml medium) the radioactivity was determined in the cold trichloroacetic acid precipitable fraction. The contribution of [^{3}H]-leucyl-tRNA was determined in the hot trichloroacetic acid precipitable fraction and did never exceed 3%.

Survival determinations

Synchronized cells, obtained by mitotic shake-off, were plated in appropriate numbers into 25 cm^{2} flasks (Greiner) and temperature treatment started at various time intervals after seeding. Colony formation was judged 5 days later after fixation and Giemsa staining. Plating efficiency ranged between 50 and 60%. In these experiments on synchronized cells, survival curves were not corrected for cellular multiplicity.

Statistical analysis

Parameters of survival curves (Do and Dq) and cell cycle progression curves (minimal time and probability constant) were calculated by least-squares analysis of the linear portion of the curves. The values of each of the parameters have been compared and tested for being significantly different by Student's-t test.

Materials

Insulin and transferrin were from Sigma, Na_2SeO_3 was from Collaborative Research. All other tissue culture components were from Gibco Grand Island Biological Co. (Grand Island N.Y.). Radioactive chemicals were obtained from Amersham International (Amersham, United Kingdom).

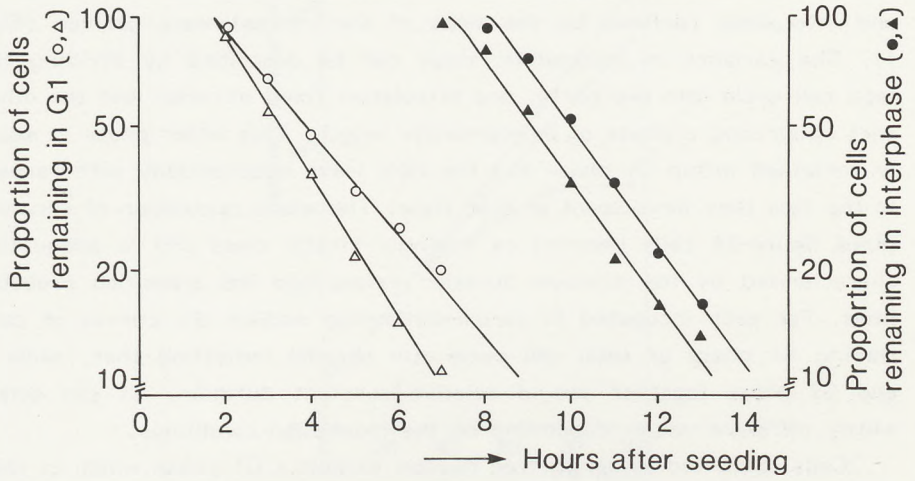

Fig. 1. Effect of serum deprivation on cell cycle progression of synchronized early G1 neuroblastoma cells. Mitotic cells were plated in serum-containing medium and 1 h. later, the medium was replaced by either fresh serum medium (Δ ▲) or serum-free medium (o ●). The proportion of cells remaining in G1 (Δ o) and interphase (▲ ●) was assayed at hourly intervals by continuous [^{3}H]-thymidine incorporation (label added 1.5 h. after plating) followed by autoradiography and by assaying cumulative mitotic indices as described in § 2. Section methods. The experiment was repeated three times.

RESULTS

Effect of serum on heat induced delay of cell cycle progression of synchronized neuro-2A cells

Mitotic Neuro-2A cells obtained by the shake-off method and plated in serum-containing medium attached and spreaded within one hour. Subsequently, serum-containing medium was exchanged for either fresh serum-containing or serum-free medium. Under these conditions the progression of cells through the cell cycle was studied by assaying the proportion of cells in G1 or in interphase (G1 + S + G2), at distinct time intervals after plating, by continuous [^{3}H]-thymidine incorporation followed by autoradiography and by assaying mitotic indices as described in the section on methods. Plotting and analysis of these data was according to Smith and Martin (24): From the data semilogarithmic plots were constructed. From the linear portion of these curves the minimum time for leaving G1 and

interphase (defined as the intersection of the linear portion of the curves with the 100 percent value) and the probability constant for leaving G1 and interphase (defined by the slope of the curves) were derived (Fig. 1). The variance in intermitotic times can be described by dividing the total cell cycle into two parts, one of relative fixed duration and the other part comprising a phase of indeterminate length. This latter phase is mostly contained within G1 phase and the cells leave exponentially with respect to the time they have spent in that state. The whole population of synchronized Neuro-2A cells behaves as a single kinetic class and is adequately characterized by the minimum duration values and the transition probabilities. For cells incubated in serum-containing medium the curves of cells leaving G1 phase or total cell cycle run parallel indicating that, while S and G2 phase together are of relative constant duration, G1 can attain widely different values depending on the incubation conditions.

Cells incubated in serum-free medium exhibit a G1 phase which is characterized by a minimum duration of 1.7 hours, and a transition probability constant of 0.13 h^{-1}. In serum-incubated cells, the minimum period for leaving G1 is similar to that of serum-free incubated cells, but the probability constant was 0.18 h^{-1}.

Cells incubated in serum-free medium were delayed for 1 hour in completing mitosis as compared to cells grown in serum-containing medium. The probability constant for leaving interphase and entering mitosis was 0.17 h^{-1}, irrespective as to whether the presence or absence of serum during cell cycle traverse.

Although serum withdrawal caused only a 1 hour delay of cells entering mitosis, the maintenance of their growth rate did require serum (Fig. 2). Moreover, the growth stimulating capacity of serum could be mimicked by growth factors. The presence of 100 ug human transferrin, 5 ug bovine pancreas insulin and 5.2 ng Na_2SeO_3 per ml DFBH stimulated to almost normal growth rate.

In a previous study (2) it has been shown that during incubation of synchronized Neuro-2A cells in serum-containing medium, a heat shock (30 minutes treatment at 42.7°C) caused an extension of the cell cycle.The effect of serum on heat induced delay of cell cycle progression of G1 phase cells is shown in Table 1. Upon incubation of heat treated G1 phase cells (1 hour after seeding) during and after the heat treatment in medium without supplements, the minimum duration for leaving G1 and interphase significantly increased ($p < 0.05$) with 4 and 2.8 hours respectively (as compared to serum-free control cells). Furthermore the probability constant for leaving G1 phase did not decrease, but for leaving interphase it

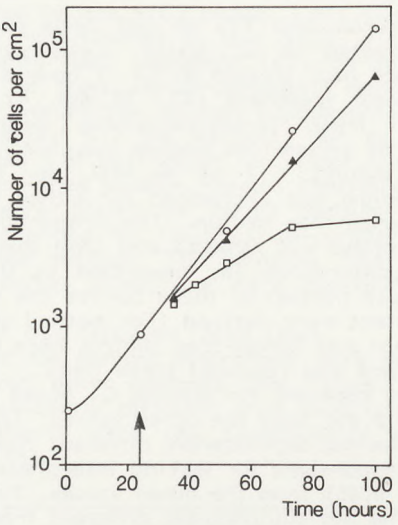

Fig. 2. Effect of serum and TIS on growth of Neuro-2A cells. Cells were plated at a density of 2.5. 10^2 cells per cm^2 in DFBH medium/10% FCS and 24 hours later (indicated by arrow) the medium was exchanged for fresh DFBH medium/10% FCS (o), DFBH medium supplemented with 100 ug transferrin/5 ug insulin/ 5.2 ng Na_2SeO_3 per ml. (▲) or DFBH medium without supplements (□). Standard errors have been omitted but are less than 10% (N = 3).

significantly decreased ($p < 0.05$) to 0.04 h^{-1} after heat treatment. The heat induced delay in leaving G1, due to increased minimum duration, was significantly diminished ($p < 0.05$) when serum was present during heat treatment, an effect which could be mimicked by TIS. The heat induced mitotic delay, due to decreased transition probability values, was also significantly diminished ($p < 0.05$) when either serum or TIS were present during heat treatment (or when serum was present after heat treatment. Data not shown). In addition the role of serum in regulating cell cycle progression was studied in heat treated late S/G2 phase cells (7 hours after seeding) (Table 2). Mitotic delay induced by a 30 min. heat treatment at 42.7°C in serum incubated late S/G2 phase cells was more than mitotic delay induced in serum incubated G1 cells. (Table 1 and 2). In contrast to G1 phase cells, the delay of heat treated serum-free incubated late S/G2 phase cells was totally due to increased minimum duration of interphase period without any significant effect on transition probability.

The presence of serum or TIS during heat treatment of G2 phase cells,

TABLE 1 Effect of serum and TIS on cell cycle progression of Neuro-2A cells heat treated in G1 phase. Cells synchronized by mitotic shake-off were heat treated in G1, 1 hour after seeding. Immediately before heat treatment (ΔT: 30 min. 42.7°C) medium was exchanged for fresh serum-containing medium (+S), serum-free medium (-S) or serum-free medium supplemented with 100 µg transferrin/5 µg insulin/ 5.2 ng Na_2SeO_3 per ml. (TIS). After heat treatment medium was exchanged for either fresh serum-containing medium or serum-free medium. The proportion of cells remaining in G1 and interphase was assayed and from these data semi-logarithmic plots were constructed (as described in the legend to Fig. 1). From the linear portion of these curves the minimum time and probability constant were derived (For method see page 75).
Data for serum and serum-free control were derived from Fig. 1.
This experiment was repeated three times.

* Probability constant for leaving G1 phase was significantly different ($p < 0.05$) from the other values. These other values were found to be not significantly different from each other ($p < 0.05$).

\+ Probability constant for leaving interphase was significantly different ($p < 0.05$) from the other values. These other values were found to be not significantly different from each other ($p < 0.05$).

	G1-PHASE		INTERPHASE	
	minimum time (h)	probability constant (h^{-1})	minimum time (h)	probability constant (h^{-1})
serum control	1.7 ± 0.1	0.18 ± 0.01*	7.2 ± 0.3	0.17 ± 0.01
heat treated in +S, recovery in +S	2.8 ± 0.1	0.13 ± 0.01	9.9 ± 0.5	0.19 ± 0.01
serum-free control	1.7 ± 0.1	0.13 ± 0.01	8.1 ± 0.2	0.17 ± 0.01
heat treated in -S, recovery in -S	5.7 ± 0.5	0.12 ± 0.01	10.9 ± 0.1	0.04 ± 0.00+
heat treated in +S, recovery in -S	2.7 ± 0.2	0.13 ± 0.01	10.3 ± 0.4	0.18 ± 0.01
heat treated in TIS, recovery in -S	3.4 ± 0.2	0.12 ± 0.01	10.5 ± 0.2	0.18 ± 0.00

TABLE 2 Effect of serum and TIS on cell cycle progression of Neuro-2A cells heat treated in late S/G2 phase. Cells synchronized by mitotic shake-off were heat treated in late S/G2, 7 hours after seeding. Immediately before heat treatment ($_{\Delta}$T: 30 min., 42.7°C) medium was exchanged for fresh serum-containing medium (+S), serum-free medium (-S) or serum-free medium supplemented with 100 $_{u}$g transferrin/5 $_{u}$g insulin/ 5.2 ng Na_2SeO_3 per ml. (TIS). After heat treatment medium was exchanged for either fresh serum-containing medium or serum-free medium. Serum control cells and serum-free control cells were plated in serum medium and 7 hours later medium was exchanged for fresh serum-containing medium, respectively serum-free medium. The minimum time and the probability constant for leaving interphase were determined as described in Table 1.

This experiment was repeated three times.

* Minimum times for leaving interphase were significantly different ($p < 0.05$) from other minimum time values. These latter values were not significantly different from each other ($p < 0.05$). Probability constants were not significantly different from each other ($p < 0.05$).

	INTERPHASE	
	minimum time (h)	probability constant (h^{-1})
serum control	7.2 ± 0.3*	0.17 ± 0.01
heat treated in +S, recovery in +S	10.7 ± 0.1	0.18 ± 0.00
serum-free control	7.6 ± 0.3*	0.17 ± 0.01
heat treated in -S, recovery in -S	12.0 ± 1.0	0.16 ± 0.01
heat treated in +S, recovery in -S	11.2 ± 0.6	0.17 ± 0.01
heat treated in TIS, recovery in -S	11.5 ± 0.6	0.17 ± 0.01

did not significantly affect the minimum duration, nor the transition probability constant as compared to those of G2 phase cells, heat treated and incubated in serum-free conditions.

Effect of serum on heat induced inhibition of protein synthesis of synchronized neuro-2A cells

Although, the protection by serum or TIS of heat induced mitotic delay has been established for G1 phase cells, the underlying mechanism is still unknown. It is of interest to consider the role of protein synthesis since various authors have reported that (i) protein synthesis was essential for progression of cells through the cell cycle (22,23,25,26), (ii) the rate of protein synthesis decreased during heat treatment (2-5) and (iii)

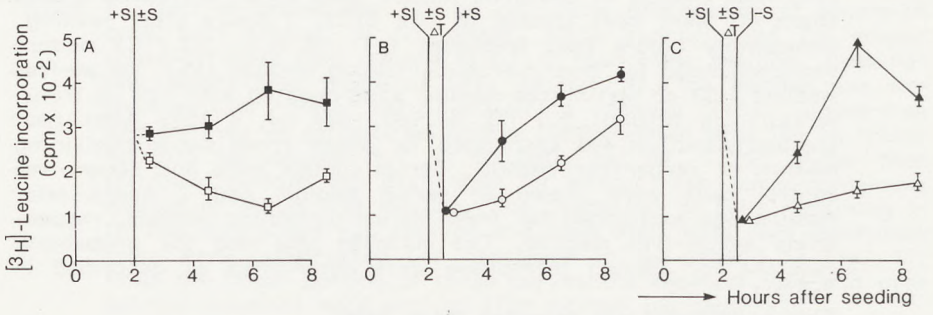

Fig. 3. Effect of serum on protein synthesis of unheated and heat treated synchronized Neuro-2A cells.
A. Synchronized cells in G1 at 2 h. after seeding were supplemented with either fresh serum (+S) medium (■) or serum-free (-S) medium (□).
B. As A, but cultures supplemented with serum (●) or serum-free (○) medium were heat treated (30 min. 42.7°C) immediately after medium change. After heat shock (Δ T) cells were incubated in serum medium.
C. As B, but after heat shock cultures supplemented with serum (▲) or serum-free (△) medium were incubated in serum-free medium.
Radioactivity was expressed per 10^5 cells. Points, means: bars, S.E. (N=3).

the mitotic delay of Neuro-2A cells heated during G1 phase could be correlated with reduction of protein synthesis of these cells, in contrast to heat treated G2 phase cells (2). Therefore we have analyzed the effect of serum in the inhibition of protein synthesis of G1 phase cells by heat treatment (30 min. at 42.7°C).

Protein synthesis was affected by heat treatment, and the decrease in the rate of protein synthesis during and after heat treatment was dependent on the presence of serum (Fig. 3). In the continuous presence of serum, the rate of protein synthesis of a synchronized cell population increased gradually. Removal of serum from G1 phase cells at 2 hours after plating resulted in a decreased rate of protein synthesis (Fig. 3a). Exposure of G1 phase cells, 2 hours after plating, to a heat shock (30 min. at 42.7°C) followed by incubation at 37°C, caused an initial inhibition of protein synthesis to 30% of control values, irrespective as to whether the presence or absence of serum during heat treatment (Fig. 3b, c). In contrast, the recovery of protein synthesis after heat treatment was dependent on the presence of serum during heat treatment. Protein synthesis

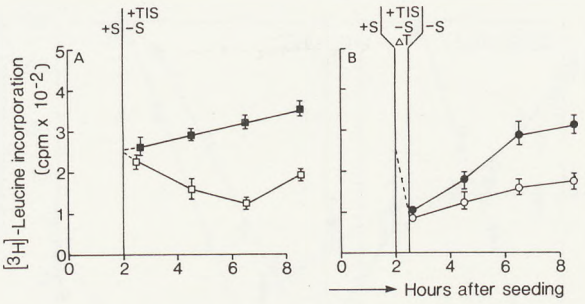

Fig. 4. Effect of TIS on heat induced alterations of protein synthesis of synchronized Neuro-2A cells.
A. Synchronized cells in G1 at 2 h. after seeding were supplemented with serum-free (-S)medium (□) or with medium containing 100 ug transferrin/5 ug insulin/5.2 ng. Na_2SeO_3 (+ TIS) per ml. medium (■).
B. As A, but cultures with serum-free (○) or +TIS (●) medium were heat treated (30 min. 42.7°C) immediately after medium change. After heat shock (ΔT) cells were incubated in serum-free medium.
Radioactivity was expressed per 10^5 cells.
Points, means: bars, S.E. (N=3).

restored at a faster rate when serum had been present during heat treatment (Fig. 3b, c). When serum was absent during heat treatment, protein synthesis recovered to serum-free control level, although protein synthesis recovered more rapidly in the presence than in the absence of serum. Furthermore, in the presence of serum during heat treatment restoration of protein synthesis to serum control values was found, irrespective as to whether the presence or absence of serum after the heat treatment.

These results demonstrate that the recovery of protein synthesis of G1 phase cells after heat shock is mainly determined by the presence of serum during the heat shock.

In addition, the effect of TIS on protein synthesis of unheated and heat treated G1 phase cells was studied. As for serum, in the continuous presence of TIS, the rate of protein synthesis of a synchronized cell population increased gradually (Fig. 4a). In heated cells (30 min. at 42.7°C), no effect of TIS on initial inhibition of protein synthesis was found (Fig. 4b). However, recovery of protein synthesis of G1 phase cells after heat shock was affected by the presence of TIS during the heat treatment. When TIS was absent during the heat treatment, protein synthesis recovered to serum-free control level. But in the presence of TIS

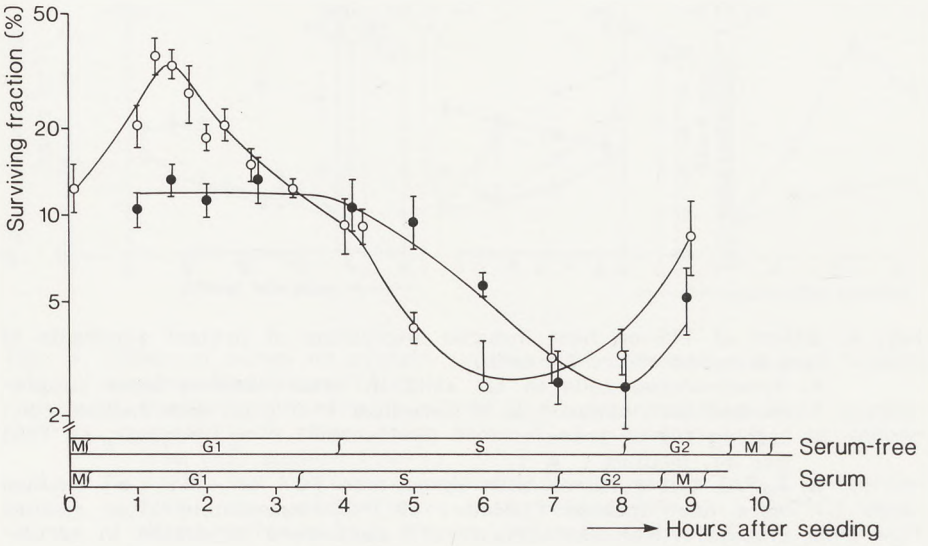

Fig. 5. Survival of synchronized Neuro-2A cells heat-treated (45 min. at 44°C) at various time intervals after seeding, during incubation in serum-containing or serum-free medium. Cells synchronized by mitotic shake-off were plated at low density, appropriate for clonal assay, in serum-containing medium and 1 hour after seeding medium was exchanged for either fresh serum-containing medium (○) or serum-free medium (●). After heat treatment medium was exchanged for fresh serum-containing medium in order to permit clone development. Points, means: bars, S.E. (N=3).

during heat treatment, a restoration to TIS control values was found.

These results demonstrate that TIS mimics the protective effect of serum on heat induced mitotic delay of G1 phase cells as well as the protective effect of serum on heat inhibited protein synthesis.

Effect of serum on heat induced killing of synchronized neuro-2A cells

The effect of heat on cell cycle extension appeared to be cell cycle dependent (2). Serum could protect cells when they were heat treated in G1. In contrast to G1 phase cells, protection by serum was absent in late S/G2 phase cells. The elevation of temperature (30 min. at 42.7°C) was sufficient to study the effects on cell cycle progression without any interference of heat induced cell death. More severe heat treatments were needed to test cell cycle dependency of heat induced cell killing. In order to study the influence of serum on heat induced cell killing, a heat

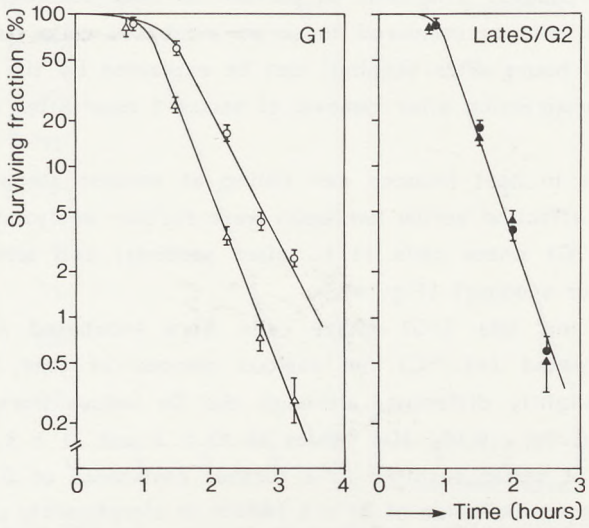

Fig. 6. Effect of serum on survival response of synchronized Neuro-2A cells continuously heated at 42.7°C for various lengths of time. Cells synchronized by mitotic shake-off were plated at low density, appropriate for clonal growth determination. In G1, (○ △) 1 hour after seeding or in late S/G2, (● ▲) 7 hours after seeding, cells were heat treated. Immediately before the start of heat treatment medium was exchanged for either fresh serum-containing (○ ●) or serum-free medium (△ ▲). After heat treatment medium was exchanged for fresh serum-containing medium in order to permit clonal development. Points, means: bars, S.E. (N=3)

treatment of 45 min. 44°C was used. The cell cycle dependency of heat induced cell killing was determined in synchronized cultures incubated in serum-free medium, 1 h. after seeding the mitotic cells.

Cell detachment was not observed in any of the cell cycle phases. When tested in synchronized cultures incubated in serum-free medium, heat sensitivity was constant during G1 phase and increased during S phase, followed by a decrease in late S/G2 phase cells.(Fig. 5)

Cells incubated in serum-containing medium demonstrated an altered pattern of heat sensitivity during cell cycle progression. In G1 phase cells, serum had a protective effect on heat induced cell killing, but this protection diminished at the G1/S boundary. The increase in heat sensitivity during S phase became manifest approximately 1-1.5 hour later in serum-free incubated cells as compared to serum incubated cells (at 65% of

cells in S). No direct effect of serum on heat sensitivity was found during late cell cycle stages. The shift in pattern of heat sensitivity of serum-free incubated cells as compared to serum-incubated cells during the late cell cycle (> 3 hours after seeding) can be explained by the 1 hour delay of cell cycle progression after removal of serum 1 hour after seeding (Fig. 1).

Differences in heat induced cell killing at various stages of the cell cycle and the effect of serum hereupon were further analyzed in a comparison between G1 phase cells (1 h. after seeding) and late S/G2 phase cells (7 h. after seeding) (Fig. 6).

When G1 and late S/G2 phase cells were incubated in serum-free medium and heated (42.7°C) for various periods of time, the survival curves were slightly different, although the Do values were not significantly different ($p < 0.05$) (Do values of 23 ± 2 and 21 ± 3 min. resp.). The presence of serum resulted in a further resistance of G1 phase cells characterized by a Do value of 31 ± 3 (which is significantly different [$p < 0.05$] from the Do value for G1 cells heated in serum-free medium). Survival curves of heat treated late S/G2 phase cells incubated in either serum-free or serum-containing medium did not have significantly different ($p < 0.05$) Do and Dq values. Apparently, in the presence of serum G1 phase cells were thermoresistent as compared to late S/G2 phase cells, due to protection of G1 cells by serum.

In that respect, protection of heat induced cell death by serum has a similar cell cycle dependence as compared to serum protection of heat induced alteration of cell cycling.

The question arose whether the protective effect of TIS as observed for heat induced cell cycle delay of G1 phase cells was also found for heat induced killing of G1 phase cells. As shown in Fig. 7, presence of TIS during heat treatment of G1 or late S/G2 phase cells did not alter heat sensitivity of cells incubated in serum-free medium. Thus the protective effect of serum on survival loss of G1 phase cells after heat treatment can not be mimicked by TIS. This conclusion was confirmed by studying the thermal sensitivity of cells during their progression through G1 and early S phase. As shown in Fig. 8, thermosensitivity of G1 phase cells incubated in TIS-supplemented medium during heat treatment (45 min. 44°C) was identical to the sensitivity of cells incubated in medium without supplements (Fig. 5).

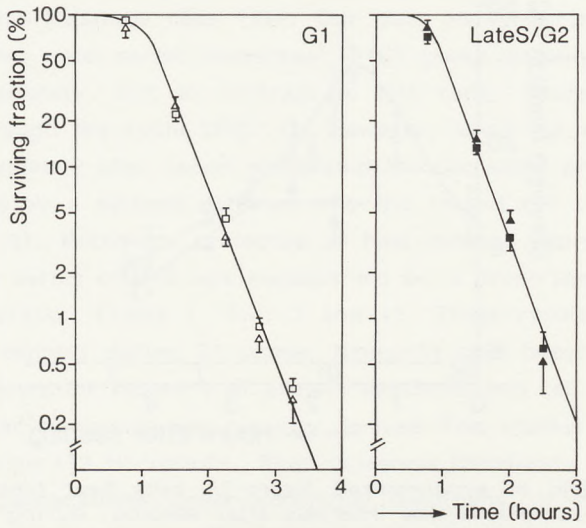

Fig. 7. Effect of TIS on survival response of synchronized Neuro-2A cells continuously heated at 42.7°C for various lengths of time. Cells synchronized by mitotic shake-off were plated at low density, appropriate for clonal growth determination. In G1 (□ △), 1 hour after seeding or in late S/G2 (■ ▲) 7 hours after seeding, cells were heat treated. Immediately before the start of heat treatment medium was exchanged for either serum-free medium supplemented with 100 µg transferrin/ 5 µg insulin/ 5.2 ng Na_2SeO_3 (TIS) per ml. (□ ■), or serum-free medium without supplements (△ ▲). After heat treatment medium was exchanged for fresh serum-containing medium. Points, means: bars, S.E. (N=3)

DISCUSSION

The data presented in this paper demonstrate that serum protects G1 phase cells for a number of heat induced cellular responses, e.g. mitotic delay (Table 1), inhibited protein synthesis (Fig. 3) and cell death (Fig. 5 and 6), in contrast to late S/G2 phase cells. Furthermore, the protection for heat induced mitotic delay and inhibited protein synthesis of G1 phase cells could be mimicked by TIS (Table 1 and Fig. 4). The effect of serum on heat induced mitotic delay is an immediate response, i.e. removal of serum caused an immediately sensitization of the cells. This protective effect of serum during heat treatment could only be observed when cells were incubated in the absence of serum after heat shock (Table 1).

In non-heated Neuro-2A cells, decrease of serum concentration hardly influences completion of the next mitosis although growth was arrested

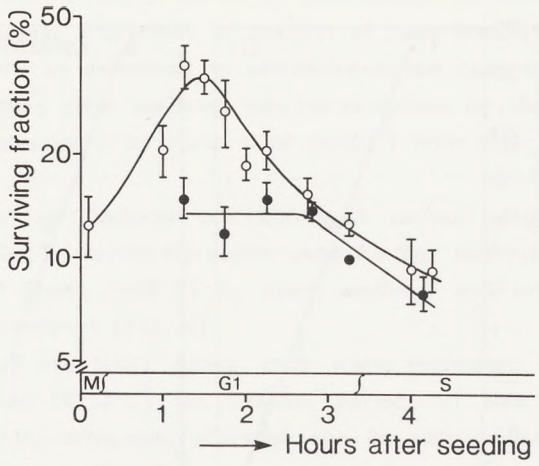

Fig. 8. Survival of synchronized Neuro-2A cells heat treated (45 min. 44°C) at various time intervals after seeding, during incubation in serum-containing medium or serum-free medium supplemented with TIS. Cells synchronized by mitotic shake-off were plated at low density, appropriate for clonal assay, in serum-containing medium, and 1.25 h. after seeding medium was exchanged for either fresh serum-containing medium (○) or serum-free medium supplemented with 100 ug transferrin/ 5 ug insulin/ 5.2 ng Na_2SeO_3 (TIS) per ml. (●). After heat treatment medium was exchanged for fresh serum-containing medium in order to permit clone development. Points, means: bars, S.E. (N=3)

afterwards (Fig. 2).

When Neuro-2A cells in G1 phase of the cell cycle, were exposed to heat immediately after transfer to serum-free medium, the kinetics of entrance into the second cell cycle were changed. Heat treatment influenced specifically in G1 phase cells the rate constant describing the function of entrance into second cell cycle. This rate constant was found to be sensitive for the presence of serum, only in G1 cells that had been heated. In contrast, the rate constant describing the entrance of G1 cells into S phase under serum-free conditions was not altered by heat, nor was it sensitive for the presence of serum in heated G1 cells. Under these conditions only the minimum duration of G1 was affected. Probably, in G1 cells, kinetics for entrance into S phase are differently influenced by heat shock as compared to kinetics for entrance into mitosis.

Some authors assume the involvement of protein synthesis in regulation of cell cycle progression. (22,23,25,26). The level of protein synthesis during serum limitation of G1 phase cells is an important signal for DNA

replication and subsequent entrance into second cell cycle as demonstrated in Reuber H35 hepatoma cells (23). Our data on synchronized Neuro-2A cells show that upon serum withdrawal in G1 phase protein synthesis decreases immediately, but in contrast to H35 cells, Neuro-2A cells still progress through the cycle (Fig. 1). However, when the cells were heat shocked immediately after serum withdrawal the decreased protein synthesis was reflected by a delayed entrance into the second cell cycle. (Table 1 and Fig. 3, 4). Moreover, protection of heat induced decrease in protein synthesis by serum or TIS was accompanied by a proportional decrease of cell cycle duration (Table 1, Fig. 3 and 4). These results indicate that upon heat treatment during G1 phase, Neuro-2A cells become more dependent upon serum for recovery of protein synthesis and cell cycle progression. A similar conclusion was recently derived from studies with confluent quiescent mouse 3T3 fibroblasts. When quiescent fibroblasts were incubated at elevated temperatures under non-lethal conditions, it was shown that they became more sensitive for epidermal growth factor with respect to progression of cells into S phase (27) This effect is dependent on the (combination of) growth factor(s) (28).

In Neuro-2A cells a higher heat dose is needed for initiating cell killing than for causing cell cycle delay and inhibition of protein synthesis. Nevertheless, a rapid effect of modulation of this heat response occurred following alteration in serum concentration. Moreover, survival of heat treated Neuro-2A cells was also modified by the cell cycle phase of the cells. In fact, cell cycle effects were small when Neuro-2A cells were incubated in serum-free medium during heat treatment. Only heat sensitivity of G1 phase cells was dependent on serum. The protective effect of serum was due to significantly ($p < 0.05$) increased Do and Dq values (Fig. 6). In contrast, TIS did not influence heat sensitivity of G1 cells (Fig. 7 and 8).

Protective effects of serum were also shown in other studies using lethal heat doses. They were demonstrated for heat induced morphological alterations of mouse 3T3 cells (29). Prostaglandin F2α markedly increased the heat resistance of cell morphology, but only when the heat was applied within a few hours after addition. This effect of growth factors was, however, small as compared to serum. Hahn (30) reported that survival of Chinese hamster (HA1) cells after heat treatment was decreased following chronically deprivation of serum. In this case protection of serum deprived cultures occurred after reincubating cells with serum containing medium for several hours.

In conclusion, our data show that serum protects G1 phase cells for heat induced changes of cell cycle progression as well as against heat induced cell death but the mechanisms involved in both phenomena seem to be different. The underlying principles responsible for the immediate protective effect of serum on heat induced alteration of proliferation capacity are under further study.

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Chapter 6

Evidence for a role of heat shock proteins in proliferation after heat treatment of synchronized mouse neuroblastoma cells

Submitted for Radiat. Res.

G. van Dongen and R. van Wijk

SUMMARY

A role for heat shock proteins (HSP's) in proliferation after heat treatment was considered in synchronized mouse neuroblastoma cells. For this purpose enhancement of HSP synthesis after heat treatment was inhibited by actinomycin D and the effect of this on cell cycle progression into mitosis and on cell survival was studied both in thermoresistant G1 and in thermosensitive late S/G2 phase cells.

In G1 phase cells expression of basal and heat induced HSP synthesis was the same as in late S/G2 phase cells, which suggests that regulation of thermoresistance throughout the cell cycle is not directly linked with HSP synthesis. HSP synthesis was enhanced after a 30 minutes treatment at temperatures of 41°C or higher. Increase of HSP synthesis after heat shock was partly suppressed by the presence of 0.1 ug/ml actinomycin D during heat treatment while 0.2 ug/ml prevented enhancement of HSP synthesis completely. Suppression of heat induced HSP synthesis by actinomycin D had a same concentration dependency in G1 and late S/G2 phase cells.

Actinomycin D potentiated induction of mitotic delay by heat treatment

(30 min. 42.5°C) but only under conditions that it actually inhibited heat induced enhancement of HSP synthesis. Also heat induced cell killing was potentiated by actinomycin D. The synergistic effect of actinomycin D and heat on induction of mitotic delay and on induction of cell killing was more pronounced in G1 phase cells than in late S/G2 phase cells. These results give evidence for a role of HSP's in proliferation after heat treatment and suggest that heated G1 phase cells are more dependent on HSP synthesis for recovery of proliferation after heat treatment than heated late S/G2 phase cells.

INTRODUCTION

Elevation of temperatures above 41°C resulted in a number of effects in cultured mammalian cells. A heat treatment could cause inhibition of DNA- (1,2) and protein synthesis (2-5) and disruption of the cytoskeleton (6-11). As long as heat treatment had no lethal effects these processes c.q. structures recovered upon reincubation at 37°C and became thermotolerant.

Furthermore cell cycle progression was delayed (2, 12-17). Mitotic delay following heat treatment was large in late S/G2 as compared to G1 phase cells. Also appearance of thermotolerance of cell cycle progression was observed (2).

Heat treatment could ultimately result in the loss of division potential and cell death (18-22). In cell survival studies, G1 phase cells were found thermoresistant as compared to S phase cells (19,22). Exposure of mammalian cells to a pulse heat shock followed by several hours recovery at normal growth temperature caused cells to become thermotolerant (23-25).

In recent years it has been shown that heat or other environmental stresses enhance the synthesis of a set of proteins, the so called heat shock proteins (HSP's) in a wide variety of cells (for reviews see 26-30). As formulated by Nover (29), a considerable number of experimental facts suggest an essential function of HSP's for protection of cells against heat damage but also for a rapid and complete recovery of normal and cellular activities after the stress period. Most arguments leading to formulation of this supposed function of HSP's came from experiments where the relationship between appearance of thermotolerance for heat induced cell death and the enhanced synthesis of HSP's was determined. In studies on fractionated hyperthermic treatment an intimate connection between formation of HSP's and development of thermotolerance was found (31-35). Moreover,

also other inducers of HSP synthesis were active in induction of thermotolerance (31,36,37).

The question can be asked whether heat shock proteins have also a function in recovery of proliferation after heat shock. Furthermore, since effects of heat on cell cycle progression and survival are cell cycle related it is interesting to determine the thermal resistance throughout the cell cycle in relation to induction of HSP synthesis.

It has been shown that induction of HSP synthesis is regulated at both the transcriptional and translational level (38,39). Inhibitors of RNA synthesis such as actinomycin D, prevented induction of HSP synthesis (40-43). Use of actinomycin D during heat shock gave us the opportunity to study cell cycle progression and cell survival of heated cells in which induction of HSP synthesis was suppressed. For this purpose we used actinomycin D in concentrations (a) sufficient to suppress induction of HSP synthesis and (b) hardly affecting cell proliferation of unheated cells. We performed this study both in thermoresistant G1 phase cells and in thermosensitive late S/G2 phase cells.

In this paper we demonstrate that inhibition of heat induced enhancement of HSP synthesis by actinomycin D coincides with a potentiation of heat induced mitotic delay and heat induced cell killing. This potentiating effect of actinomycin D was more in G1 as compared to late S/G2 phase cells. Heat induced enhancement of HSP synthesis was similar in G1 and late S/G2 phase cells but the data suggest that heated G1 phase cells are more dependent on HSP synthesis for recovery of proliferation after heat treatment than heated late S/G2 phase cells.

MATERIALS AND METHODS

Cell culture

Mouse neuroblastoma N2A cells were routinely grown at 37°C as subconfluent monolayer cultures in 150 cm^2 plastic Falcon flasks. The medium consisted of Dulbecco's modified Eagle's medium with 10 mM Hepes (pH 7.6) supplemented with 10 % foetal calf serum. Synchronous cultures were obtained by performing a mitotic shake-off of a subconfluent monolayer. In this procedure 20 shakes (2 shakes/s) with fresh medium were used to detach mitotic cells. The population of cells detached was approximately 6 % of the total population (and 70 % of the number of mitotic cells present) and consisted of 95 % cells in mitosis, i.e. metaphase or doublet (telophase) stage. Subconfluent monolayer cultures underwent a shake proce-

dure only once. These cells were plated at a density of 4.10^3 cells/cm². The average cell cycle duration was about 11.5 h. and consisted of a 3 h. G1, 5 h. S, 2.5 h. G2 and 1 h. M period. Analysis of durations of the cell cycle phases were performed by pulse- and continuous [^{3}H]-thymidine incorporation followed by autoradiography and by determination of mitotic indices as described previously (2).

Entrance into mitosis

Entrance of synchronized mitotic cells into their second cell cycle was determined microscopically as described before (2). According to this procedure 250 cells were counted at intervals of 1 hour in 5-7 randomly chosen microscope fields using a phase contrast microscope and the number of mitotic cells in a doublet stage was determined. The standard deviations in the counting of the individual microscope fields was not greater than 8 %. This method of counting has been applied because otherwise it is difficult to distinguish after heat treatment, the few detached interphase cells from cells in the pro-metaphase of mitosis. Furthermore, duration of anaphase and telophase was hardly influenced by heat treatment (2). The number of cells which had entered the second cell cycle was derived by accumulation of the hourly determined amount of doublet cells.

Heat treatment

For heat treatment the culture flasks were immersed in a waterbath. The heater was a circulating thermomix 1420 (Braun A.G., Melsungen, F.R.G.) provided with a proportional control of heating power giving stable temperature levels of ± 0.02°C within the range used.

Labeling of proteins and SDS-polyacrylamide gel-electrophoresis

After heat treatment the culture medium was removed and replaced by medium without methionine. Subconfluent cell cultures (3.10^4 cells/cm^2) were labeled at 37°C for 3 hours with [^{35}S]-methionine (S.A.: 250 uCi/mmol, Amersham) (7 uCi/ml). The medium was subsequently removed and the cells were washed five times with cold phosphate buffered saline (PBS). For analysis by one-dimensional SDS-polyacrylamide gel electrophoresis, the cells were solubilized in sample buffer (5 % SDS, 10 % β-mercaptoethanol, 0.0015 % bromophenol blue, 15 % glycerol and 125 mM Tris-HCl, pH 6.8). The samples were stored in sample buffer at -20°C prior to electrophoresis. SDS-polyacrylamide gels were prepared according to the procedure of Laemmli (44). Molecular mass markers used included

phosphorylase (92.000), bovine serum albumine (67.500), ovalbumine (45.000), chymotrypsinogen (25.000) and cytochrome C (12.500) (BioRad). Radioactive gels were treated with En^{3}Hance (New England Nuclear) and exposed to Kodak S.B. panoramic dental X-ray film.

RNA synthesis

The rate of RNA synthesis was determined by [^{3}H]-uridine incorporation. After labeling for 25 min. with [^{3}H]-uridine (1 uCi/ml medium: S.A. 43 Ci/mol) the radioactivity was determined in the cold trichloroacetic acid-precipitable fraction.

Survival determination

Synchronized cells, obtained by mitotic shake-off, were plated in appropriate numbers into 25 cm^2 flasks (Greiner) and temperature treatment started at various time intervals after seeding. Colony formation was judged 5 days later after fixation and Giemsa staining. Plating efficiency ranged between 50 and 60%. In these experiments on synchronized cells, survival curves were not corrected for cellular multiplicity that occurred immediately after plating.

Materials

Actinomycin D was obtained from Sigma. All tissue culture components were from Gibco Grand Island Biological Co. (Grand Island, N.Y.). Radioactive chemicals were obtained from Amersham International (Amersham, United Kingdom).

RESULTS

Induction of heat shock protein synthesis in synchronized Neuro-2A cells.

In exponentially growing Neuro-2A cells, heat treatment (30 min. 41°C, 42°C or 43°C) enhanced the synthesis of heat shock proteins with apparent molecular masses of 68.000, 70.000, 84.000 and 100.000 Dalton, and enhancement increased with heat shock temperature (Fig. 1). Basal synthesis levels of HSP 84 and HSP 100 were high and enhancement of synthesis after heat treatment was relatively small.

Heat sensitivity is dependent on cell's position in the cell cycle. In cell survival and mitotic delay studies, late S/G2 phase cells were found thermosensitive as compared to G1 phase cells (2,22). The data presented in Fig. 2 indicate that alteration of heat sensitivity during the cell cycle was not

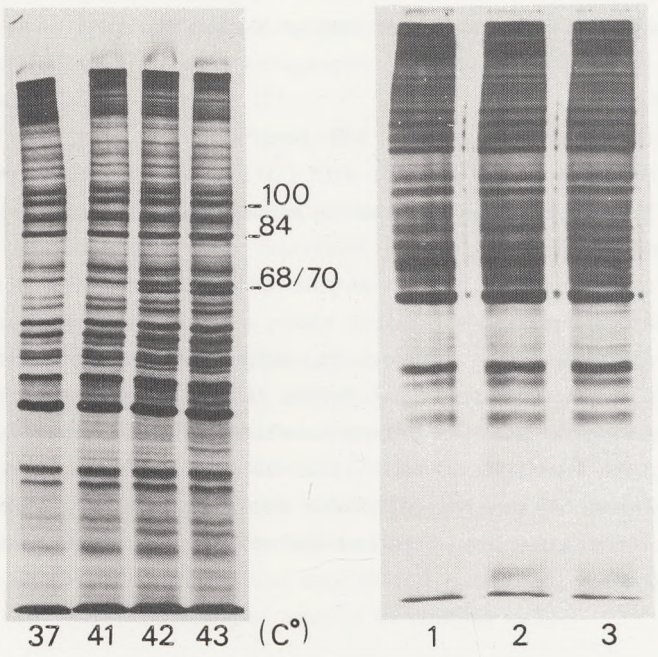

Fig. 1. Polypeptide composition of control and heat shocked (30 min. 41°C, 42°C or 43°C) neuroblastoma cells. Immediately after heat treatment cells were incubated at 37°C in the presence of [^{35}S]-methionine for 3 h. The apparent molecular weights (x 10^{-3}) of the HSP's are indicated on the right.

Fig. 2. Polypeptide composition of synchronized mouse neuroblastoma cells. Cells were synchronized by mitotic shake-off and incubated with [^{35}S]-methionine for 1 h. starting in G1 2 h. after seeding (1), mid S 5 h. after seeding (2) or in late S/G2 9 h. after seeding (3).

correlated with alteration of HSP synthesis: protein synthesis pattern of synchronized Neuro-2A cells was invariable throughout the cell cycle as determined by one-dimensional polyacrylamide gel-electrophoresis. Furthermore, induction of HSP synthesis by heat shock did not correlate with the cell cycle phase dependent heat sensitivity. Fig. 3 illustrates HSP synthesis after heat shock (30 min. 43°C) of G1 and late S/G2 phase cells: irrespective cell cycle phase, synthesis of HSP 68 and 70 remained elevated for 6 and 8 hours respectively. Also the kinetics of HSP synthesis after

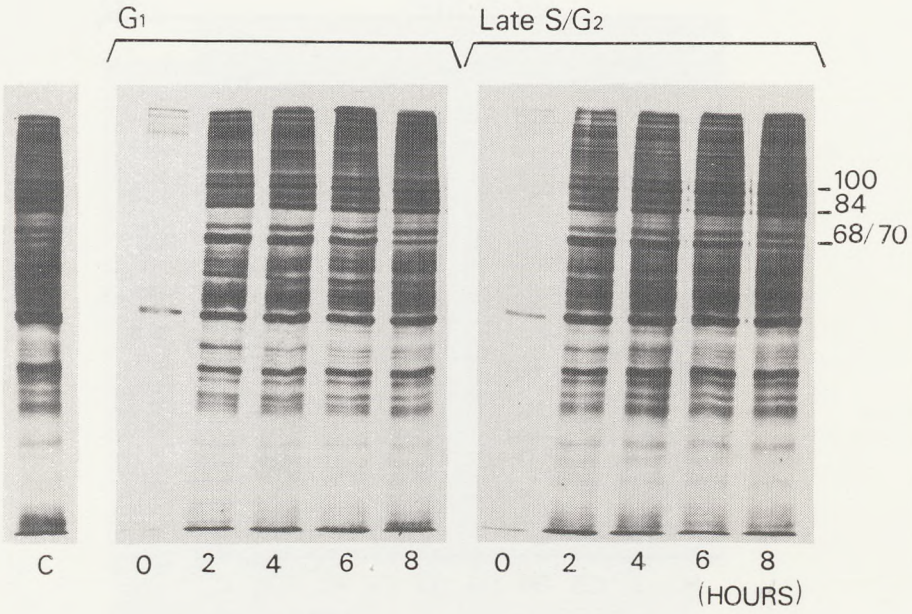

Fig. 3. Kinetics of heat induced enhancement of HSP synthesis in synchronized Neuro-2A cells. Cells synchronized by mitotic shake-off were heated (30 min. 43°C) in G1 2 h. after seeding or late S/G2 phase 9 h. after seeding and at various times after heat treatment cells were pulse-labeled with [^{35}S]-methionine for 1 h. at 37°C. The apparent molecular weights (x 10^{-3}) of HSP's are indicated on the right; Control (C).

other heat treatment protocols (30 min. 41°C, 42°C or 44°C) was the same for G1 and late S/G2 phase cells. (Data not shown). These results suggest that regulation of thermoresistance throughout the cell cycle is not directly linked with HSP synthesis.

Suppression of heat induced HSP synthesis by actinomycin D.

As shown by Fig. 1 heat treatment for 30 min. at 42°C or 43°C resulted in an extensive enhancement of HSP synthesis. We studied the effect of actinomycin D at concentrations ranging from 0.05 to 1 ug/ml on induction of HSP synthesis by a heat treatment of 30 min. at 42.5°C. Enhancement was partly inhibited when actinomycin D in a concentration of 0.1 ug/ml was added before heating and remained present during the heat (Fig. 4). Actinomycin D present in a concentration of 0.2 ug/ml or higher

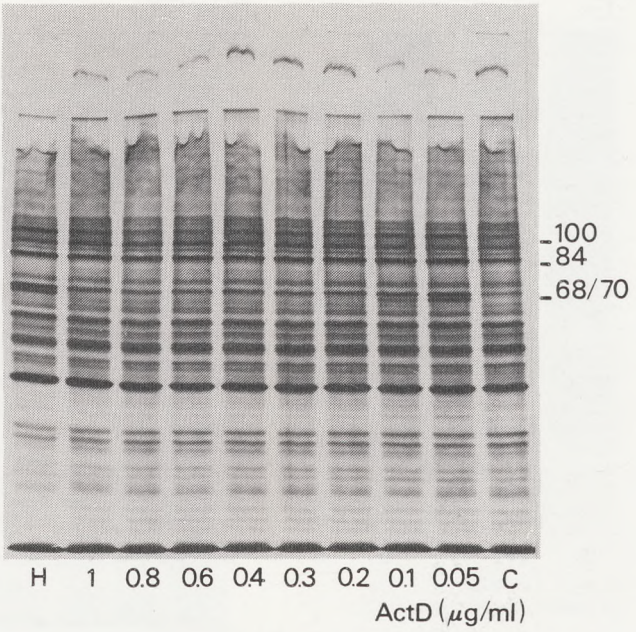

Fig. 4. Effect of actinomycin D on enhancement of HSP synthesis after heat treatment of Neuro-2A cells. Actinomycin D was added 15 min. before heat treatment (30 min. 42.5°C) to a final concentration ranging from 0.05 to 1 ug/ml medium. After heat treatment actinomycin D containing medium was removed, cells were washed two times with PBS and they were subsequently incubated for 3 h. at 37°C in medium containing [^{35}S]-methionine. The apparent molecular weights of HSP's are indicated on the right; Control (C); Cells heat-shocked without actinomycin D (H).

totally prevented elevation of HSP synthesis. Heat induced HSP synthesis was equally sensitive for actinomycin D in heated G1 and heated late S/G2 phase cells (Fig. 5). These data allow a study on the effect of heat on cell cycle progression under conditions where HSP synthesis after heat treatment is suppressed.

Effect of actinomycin D on heat induced cel· cycle delay of synchronized Neuro-2A cells.

In earlier studies we demonstrated that heat treated Neuro-2A cells had a cell cycle progression delay (2). Now we studied the effect of heat on mitotic delay under conditions that enhancement of HSP synthesis was

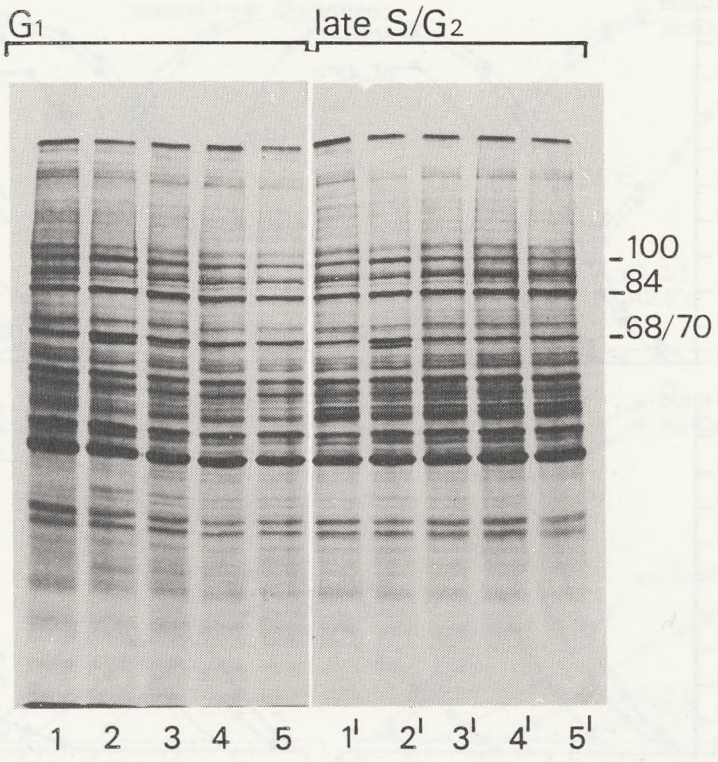

Fig. 5. Effect of actinomycin D on enhancement of HSP synthesis after heat treatment of synchronized Neuro-2A cells. Cells synchronized by mitotic shake-off were treated in G1 2 h. after seeding or late S/G2 phase 9 h. after seeding. Actinomycin D was added 15 min. before heat treatment (30 min. 42.5°C) to final concentrations of 0.1, 0.2 or 0.3 ug/ml medium. After treatment actinomycin D containing medium was removed, cells were washed two times with PBS and they were subsequently incubated for 3 h. at 37°C in medium containing [^{35}S]-methionine.
lane 1, 1': 37°C lane 3, 3': 42.5°C + 0.1 ug/ml act. D
lane 2, 2': 42.5°C lane 4, 4': 42.5°C + 0.2 ug/ml act. D
lane 5, 5': 42.5°C + 0.3 ug/ml act. D

suppressed by actinomycin D. A population of mitotic Neuro-2A cells isolated by shake-off showed an increase of mitotic indices values at 10 h. Fig. 6A shows the cumulative values of mitotic indices assayed every hour and 50% of the cells had entered the second cell cycle at 11.7 h. Treatment of G1 phase cells with either actinomycin D (45 min. 0.1 or 0.2 ug/ml) or heat (30 min. 42°C or 42.5°C) resulted in a mitotic delay which never

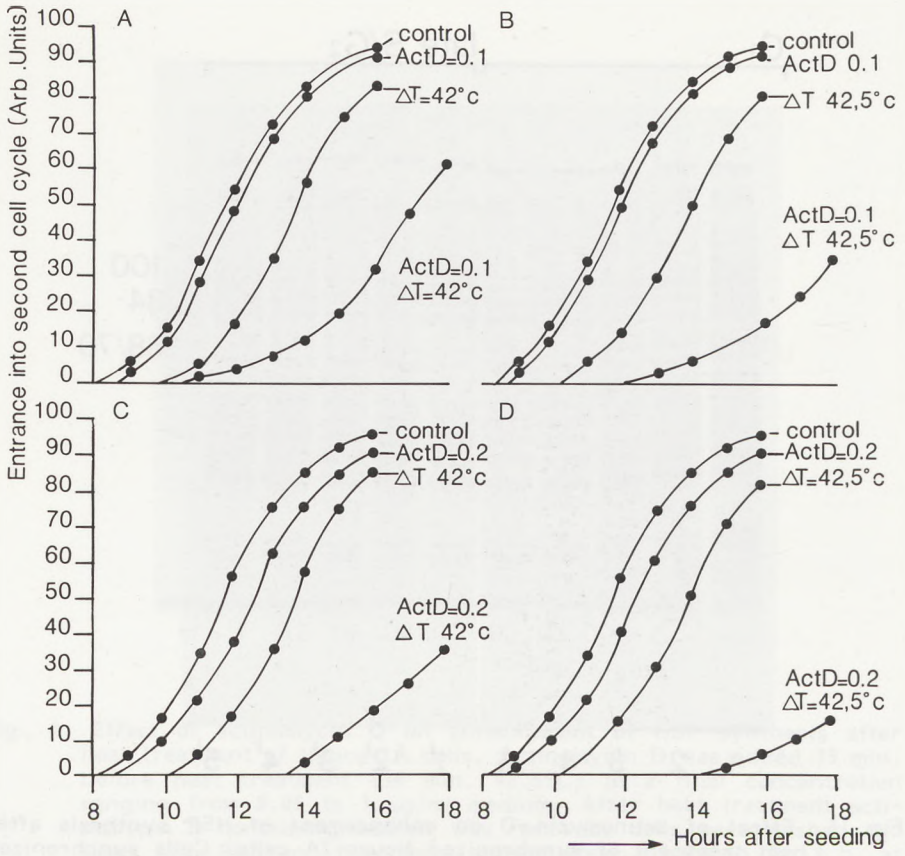

Fig. 6. Effect of treatment with actinomycin D and/or heat on mitotic delay of Neuro-2A cells treated in G1 phase. Cells were synchronized by mitotic shake-off. G1 phase cells, 2 h. after seeding were treated with actinomycin D (45 min. 0.1 or 0.2 ug/ml) and or heat (30 min. 42°C or 42.5°C). In case of combined treatment actinomycin D was added 15 min. before initiation of heat treatment. After actinomycin D treatment medium was removed, cells were washed two times with PBS and fresh medium was added. Concentration of actinomycin D (ug/ml) and temperature of heat shock (°C) are indicated in the figure. Frequency of double-ball cells was determined in duplicate cultures. The experiment was repeated three times.

exceeded 2.3 hours (as determined from time that 50% of the cells were in mitosis). After combined treatment with actinomycin D (0.1 ug/ml) and heat (42°C) cell cycle was elongated with 5.4 hours (Fig. 6A). When in this combined treatment the concentration of actinomycin D and/or temperature increased, cell cycle duration further extended. Probably in G1 phase

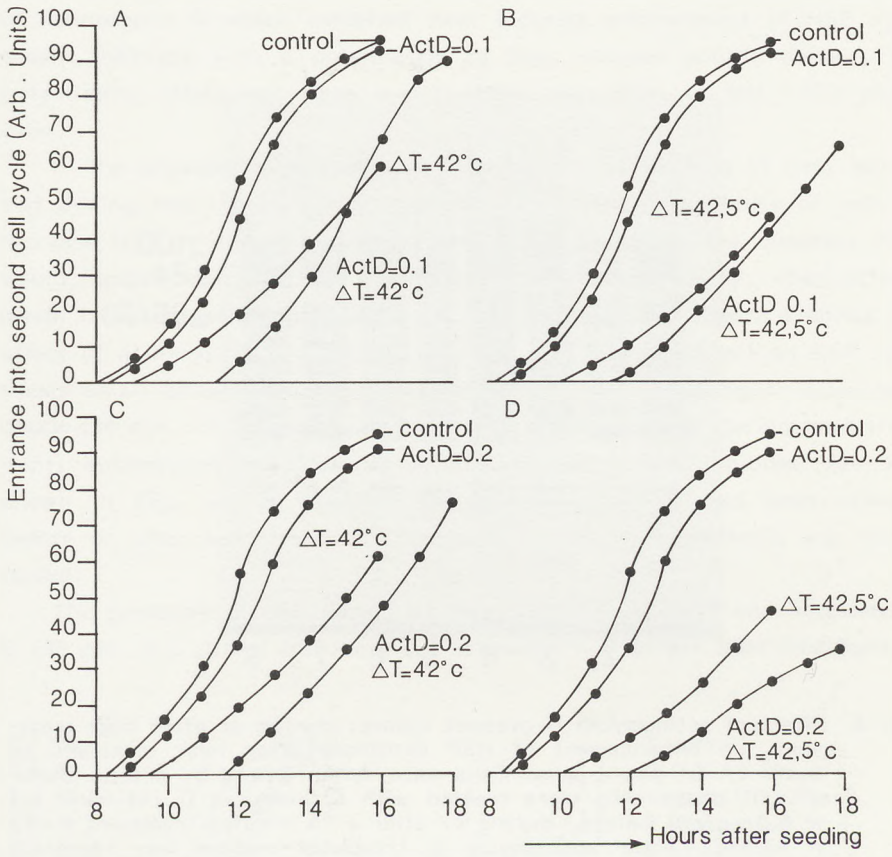

Fig. 7. Effect of treatment with actinomycin D and/or heat on mitotic delay of Neuro-2A cells treated in late S/G2 phase. Cells were synchronized by mitotic shake-off. Late S/G2 phase cells 9 h. after seeding, were treated with actinomycin D (45 min. 0.1 or 0.2 ug/ml) and/or heat (30 min. 42°C or 42.5°C). In case of combined treatment actinomycin D was added 15 min. before initiation of heat treatment. After actinomycin D treatment medium was removed, cells were washed two times with PBS and fresh medium was added. Concentration of actinomycin D (ug/ml) and temperature of heat shock (°C) are indicated in the figure. Frequency of double-ball cells was determined in duplicate cultures. The experiment was repeated three times.

cells, actinomycin D and heat, in all used combinations, extended cell cycle duration in a synergistic fashion. Similar experiments were performed with late S/G2 phase cells. As shown in Fig. 7 extension of cell cycle duration caused by actinomycin D in these cells was essentially the same as for G1

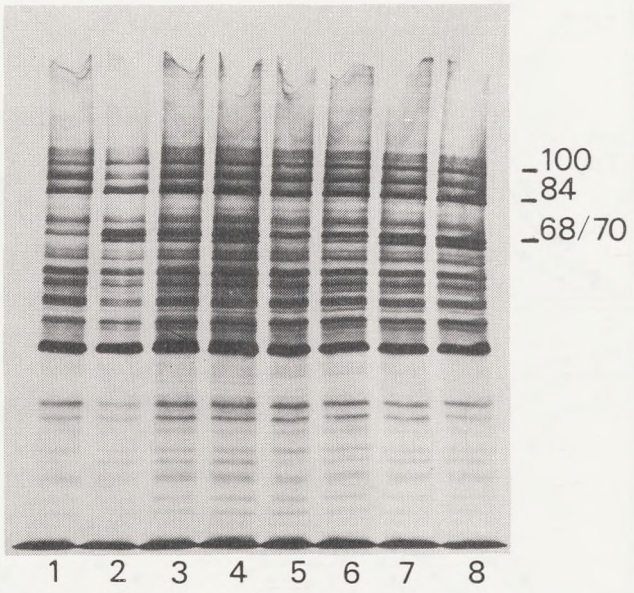

Fig. 8. Effect of actinomycin D present before, during or after heat treatment, on enhancement of HSP synthesis after heat treatment of Neuro-2A G1 phase cells. Cells were synchronized by mitotic shake-off. G1 phase cells were treated with actinomycin D (45 min. 0.1 or 0.2 ug/ml) before, during or after a 30 minutes treatment (ΔT) at 42.5°C. After actinomycin D treatment medium was removed, cells were washed two times with PBS and fresh medium was added. These cells were incubated in the presence of [^{35}S]-methionine for 3 h. The next protocols were performed indicated by time (h.) after seeding.

lane 1: control
lane 2: Δ T: 2.00-2.30
lane 3: act. D (0.2): 2.30-3.15 + Δ T: 2.00-2.30
lane 4: act. D (0.1): 2.30-3.15 + Δ T: 2.00-2.30.
lane 5: act. D (0.2): 1.45-2.30 + Δ T: 2.00-2.30.
lane 6: act. D (0.1): 1.45-2.30 + Δ T: 2.00-2.30.
lane 7: act. D (0.2): 1.15-2.00 + Δ T: 2.00-2.30.
lane 8: act. D (0.1): 1.15-2.00 + Δ T: 2.00-2.30.

phase cells. But heat induced cell cycle delay was more as compared to G1 phase cells. Furthermore in late S/G2 phase cells a combination of actinomycin D and heat disturbed progression into mitosis in a synergistic fashion but only when actinomycin D concentration was 0.2 ug/ml and heat temperature was 42.5°C (Fig. 7D). Under the other treatment conditions

actinomycin D and heat had additional effects. Apparently, concentrations of actinomycin D which inhibited heat induced enhancement of HSP synthesis, coincide with a potentiation of heat induced mitotic delay. This potentiating effect was more in G1 phase cells than in late S/G2 phase cells.

In the previous experiments actinomycin D was present 15 min. before and during heat shock only. This resulted in the incapability of cells to increase HSP synthesis after heat treatment. We asked the question what would happen with (a) HSP synthesis and (b) mitotic delay, when actinomycin D was present either before or after heat shock. Figure 8 shows the effect of actinomycin D treatment (45 min. 0.1 or 0.2 ug/ml) on HSP synthesis of G1 phase cells when present either before, during or after heat shock (30 min. 42.5°C). When actinomycin D was present during heat treatment, subsequent enhancement of HSP synthesis was inhibited (as also shown in Fig. 4). In contrast, when actinomycin D had been present before or after heat treatment, enhancement of HSP synthesis was unaffected.

The protocols of treatment with heat (30 min. 42.5°C) and actinomycin D (45 min. 0.2 ug/ml) including (a) actinomycin D before heat treatment,

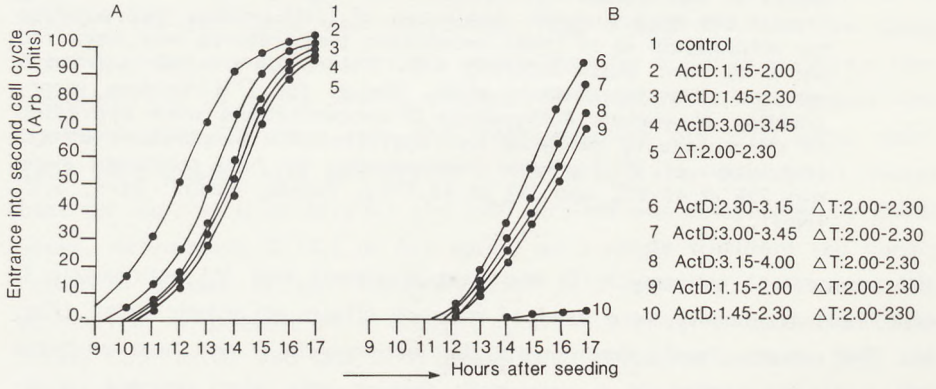

Fig. 9. Effect of actinomycin D present before, during or after heat treatment on cell cycle progression into mitosis of Neuro-2A cells treated in G1 phase. Cells were synchronized by mitotic shake-off. G1 phase cells were treated with actinomycin D (45 min. 0.2 ug/ml) before, during or after a 30 minutes heat treatment at 42.5°C. After actinomycin D medium was removed, cells were washed two times with PBS and fresh medium was added. Time (hours after seeding) of actinomycin D treatment and heat treatment (Δ T) are indicated in the figure. Frequency of double-ball cells was determined in duplicate cultures. The experiment was repeated three times.

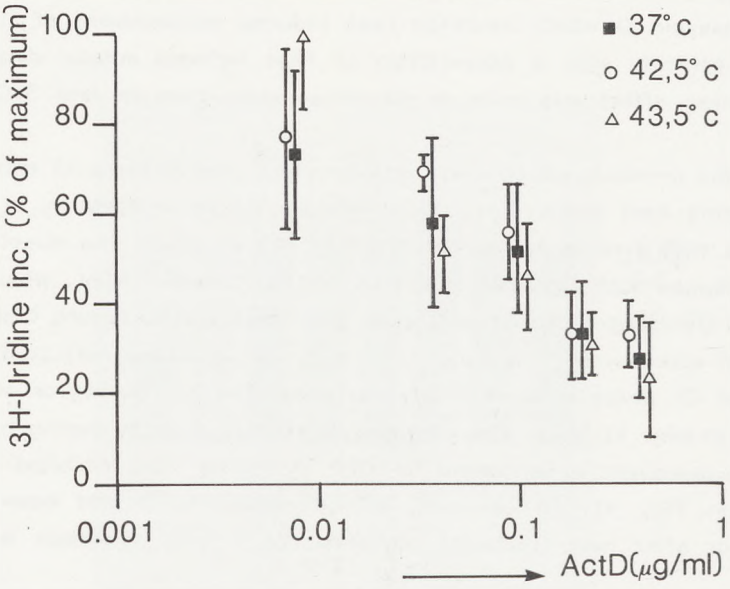

Fig. 10. Actinomycin D sensitivity of [^{3}H]-uridine incorporation by Neuro-2A cells as function of incubation temperature. 15 minutes before temperature treatment (30 min. 37°C, 42.5°C or 43.5°C) actinomycin D was added to the cells to final concentration ranging from 0.008 to 0.4 ug/ml. Inhibition of [^{3}H]-uridine incorporation by actinomycin D at those incubation temperatures was measured by a 25 min. pulse labeling with [^{3}H]-uridine which started 5 minutes after temperature shift. Values for [^{3}H]-uridine incorporation at various actinomycin D concentrations were expressed as percentage of maximum i.e. corrected for temperature effect. (Inhibition of [^{3}H]-uridine incorporation by heat treatment alone was 23% at 42.5°C and 35% at 43.5°C). Points, means: bars, S.E. (N=3).

(b) concomitant actinomycin D and heat treatment and (c) actinomycin D after heat treatment, were used to measure effects on mitotic delay (Fig. 9). The results clearly demonstrate that actinomycin D and heat inhibited cell cycle progression in a synergistic fashion only when present concomitantly. In this case cell cycle duration was extended with at least 6 hours.

It is likely to suggest that combined treatment with actinomycin D and heat leads to such large cell cycle extension as a result of specific effects of actinomycin D on the synthesis of certain proteins. Several other explanations have been considered. In a previous study (2) cell cycle extension was caused by inhibition of protein synthesis with cycloheximide. We there-

fore tested whether actinomycin D might cause this large cell cycle extension by potentiating heat inhibited protein synthesis. However as measured by [^{3}H]-leucine incorporation, no effect of actinomycin D (0.2 ug/ml) on heat (30 min. 42.5°C) induced decrease of protein synthesis was found (Data not shown). Another explanation of these observations might be that in a heated cell, transport and/or binding of actinomycin D was altered. We considered this possibility by studying the effect of various concentrations of actinomycin D on [^{3}H]-uridine incorporation into RNA, in heated (30 min. 42.5°C or 43.5°C) and unheated cells. As shown by Fig. 10, inhibition of [^{3}H]-uridine incorporation by actinomycin D depended on its concentration but this dependency was not altered by incubation temperature. These data indicate that the large mitotic delay after concomitant treatment with actinomycin D and heat as shown in Fig. 6 and 9 is evidently due to a specific effect of actinomycin D on heat induced alterations of gene expression.

Effect of actinomycin D on heat induced cell killing of synchronized Neuro-2A cells

Under several conditions that inhibited the enhancement of HSP synthesis after heat treatment, the capacity of a heated cell to progress through the cell cycle was diminished (Fig. 6 and 7). Here we study whether under these conditions the possibilities for a cell to survive a heat treatment and to divide became affected. This study was performed with thermoresistant G1 phase cells and thermosensitive late S/G2 phase cells. Cells were treated with actinomycin D (45 min. 0.1 or 0.2 ug/ml) and/or heat (30 min. 41°C or 42.5°C) and cell survival was established by clonal assay. Actinomycin D (0.1 or 0.2 ug/ml) as a single treatment did hardly result in a loss of cell survival (Fig. 11). However, treatment with actinomycin D present in a concentration of 0.3 ug/ml caused 25% cell killing both in G1 and late S/G2 phase cells (Data not shown). Fig. 11A shows that the survival response of G1 phase cells after treatment with actinomycin D and/or heat is a function of actinomycin D concentration and heat shock temperature. The data indicate that actinomycin D and heat affected cell survival in a synergistic fashion.

A similar experiment was done in late S/G2 phase cells. Also in late S/G2 cells actinomycin D and heat performed a synergistic action (Fig. 11B). But synergism was low as compared to G1 phase cells.

These results demonstrate that the specific effect of actinomycin D on heated cells, as monitored by suppression of HSP synthesis, coincides with

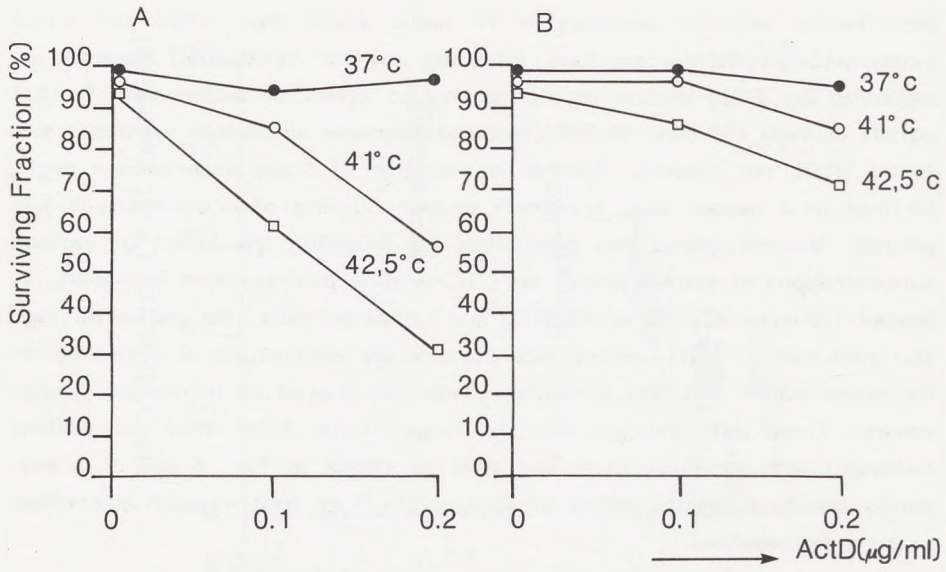

Fig. 11. Effect of a combined actinomycin D/heat treatment on survival response of Neuro-2A cells treated in G1 phase or late S/G2 phase. Cells were synchronized by mitotic shake-off. G1 phase cells, 2 h. after seeding (A) or late S/G2 phase cells, 9 h. after seeding (B), were treated with actinomycin D (45 min. 0.1 or 0.2 ug/ml) and/or heat (30 min. 41°C or 42.5°C). In case of combined treatment actinomycin D was added 15 min. before initiation of heat treatment. After actinomycin D treatment medium was removed, cells were washed two times with PBS, and fresh medium was added. Temperature of heat shock is indicated in the figure. Points, means: Standard errors have been omitted but are less than 6%.

(a) extension of cell cycle duration and (b) enhancement of cell killing. This impairement was larger in heated G1 than in heated late S/G2 phase cells.

DISCUSSION

Heat treatment of neuro-2A cells resulted in an extension of cell cycle duration. Mitotic delay was more for heated late S/G2 phase cells as compared to heated G1 phase cells. Actinomycin D potentiated heat induced mitotic delay. This effect was more in G1 phase cells as compared to late S/G2 phase cells.

Under the conditions actinomycin D potentiated heat induced mitotic delay, also heat induced cell killing was potentiated. Also this effect was

more for G1 phase cells as compared to late S/G2 phase cells.

A synergistic effect on mitotic delay was only observed when actinomycin D was present during heat treatment. When actinomycin D was present before or after heat treatment, neither heat induced mitotic delay nor heat induced HSP synthesis was affected (Fig. 8 and 9). These data indicate that actinomycin D (0.1 or 0.2 μg/ml) is reversibly bound under these conditions and that potentiation by actinomycin D is probably not caused by inhibition of RNA synthesis per sé. The observation that actinomycin D present during heat treatment does not potentiate heat inhibited [^{3}H]-uridine incorporation (Fig. 10) supports this latter conclusion.

These data suggest that one or more of the HSP's have a role in cellular processes needed for cell cycling and survival after heat shock. In general this hypothesis has been supported by several types of experimental data. A relationship between heat resistance and HSP synthesis was found in mutant cell lines. Laszlo and Li (45) isolated heat resistant variants of Chinese hamster fibroblasts and demonstrated that basal synthesis of HSP 70 was enhanced in these variants. Furthermore, induction of HSP 70 occurred at a faster rate while the maximum levels achieved were relatively higher and occurred sooner. Iida and Yahara (46) described enhanced HSP synthesis levels in heat resistant mutants of yeast. Also in studies on fractionated hyperthermia an intimate connection between increase of HSP levels and increase of thermoresistance was found (31-35).

However, in this paper we show that throughout the cell cycle, heat sensitivity and HSP synthesis are not directly related. This can be illustrated by two sets of data. In the comparison between thermoresistant G1 and thermosensitive late S/G2 phase cells no differences in basal- or heat induced HSP synthesis were observed. Furthermore, when enhancement of HSP synthesis after heat shock was suppressed by actinomycin D, heat induced mitotic delay and heat induced cell killing extended, but those effects were larger in heated G1 than in heated late S/G2 phase cells. This may indicate that the role of HSP's in protection against heat damage is more pronounced in heated G1 phase cells as compared to late S/G2 phase cells.

These data can be explained in several ways. An explanation might be that HSP's synthesized by G1 phase cells are different from those synthesized by late S/G2 phase cells. HSP's were found to be phosphorylated (47,48), methylated (49,50), glycosylated (51) and/or ADP-ribosylated (52), but it is still unknown whether modification of these proteins is related to cell cycle. Another interpretation of these results is that a

target responsible for heat killing (and/or occurrence of mitotic delay) and protected by HSP's, is expressed to a different extent throughout the cell cycle.

Omar and Lanks (53) also demonstrated that HSP synthesis is not necessarily correlated with heat resistance and they concluded that also other factors might correlate with heat resistance. Thus in the comparison between normal and Simian Virus 40 transformed mouse embryo cells they found that the higher intrinsic resistance of normal cells to killing by heat was not directly related to basal HSP synthesis or to the degree to which synthesis of these proteins was induced following exposure to hyperthermia.

The data presented in this paper may provide a framework for further investigation of the role of HSP's in cell proliferation after heat shock and may be a clue for exposition of the mechanisms upon which HSP's operate.

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Chapter 7

Increase of thermoresistance after growth stimulation of resting Reuber H35 hepatoma cells: alteration of nuclear characteristics, non-histone chromosomal protein phosphorylation and basal heat shock protein synthesis

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SUMMARY

In this paper we demonstrate increase of thermoresistance of resting Reuber H35 cells upon growth stimulation by serum containing medium: late G1/early S phase cells were thermoresistant as compared to Go phase cells. Increase of thermoresistance during early cell cycle runs parallel with increased tolerance of structural and molecular properties of the cell nucleus. Nuclear shape and chromatin texture became thermotolerant as determined by geometric and densitometric analysis of Feulgen stained nuclei. Moreover, increased tolerance was demonstrated by means of the capability for endogenous phosphorylation of isolated non-histone chromosomal proteins (NHCP's). We speculate about the molecular basis for this increased thermoresistance after growth stimulation and make a comparison with induction of 'acquired thermotolerance' as has been observed in studies on fractionated hyperthermia. Both after growth stimulation and after heat shock, an increase of endogenous phosphorylation capacity of isolated NHCP's was observed while a main enhancement of phosphorylation was found for a NHCP of Mr 95.000. Moreover, the basal synthesis of proteins inducible by heat shock (heat shock proteins) and indicated as HSP 65, HSP 68 and HSP 84 was enhanced in thermoresistant late G1/early S phase cells as compared to thermosensitive Go phase cells. A role for chromatin structuring, NHCP phosphorylation and HSP's in regulation of thermosensitivity and cell cycling is discussed.

INTRODUCTION

Hyperthermic killing of mammalian cells is cell cycle dependent (1-4). The mechanism behind heat induced cell killing and its relation to cell cycle position are still unknown. There is evidence that damage within the chromatin is responsible for cell death (5). A number of heat induced changes in nuclear functions and structures have been described: DNA synthesis is rapidly inhibited by heat (6-8), DNA strand breaks have been observed to develop with time after heating (9), the amount of non-histone proteins, tightly associated with the nucleus, increased (10,11), while also changes in the heterochromatin and the granular material of the nucleolus (12-14) and the flatness of the nucleus and its chromatin structuring have been observed (15). Furthermore, hyperthermic treatment of cells resulted in alterations in the degree of phosphorylation of isolated non-histone chromosomal proteins (12).

A variety of methods have been used to study the structure of chromatin in relation to cell cycle. Alteration of thermal denaturation profiles of chromatin prepared from cells at various cell cycle phases were observed (16). Structural changes of chromatin were also detected in situ by differential staining of chromatin-DNA by Feulgen reaction (17), acridine orange (18) or ethidium bromide (19). By use of these methods, characteristic variations in nuclear morphology have been shown to occur after growth stimulation (20) and during cell cycle (21,22). One would expect that staining methods can be used to detect alterations in thermal sensitivity of chromatin structure.

It is becoming increasingly apparent that non-histone chromosomal proteins play an important role in dictating structural and functional properties of the eukaryotic genome. Quantitative and qualitative differences in non-histone chromosomal proteins have been correlated with alterations in the biological status of cells and tissues. Changes in the composition and metabolism of various molecular weight classes of these proteins accompany changes of the physiological state of cells e.g. stimulation of cell proliferation (23,24) and progression through the cell cycle (25,26).

A number of studies revealed changes in phosphorylation of NHCP's which correlated with changes of these physiological conditions (27,28, 29). Phosphorylation of NHCP's caused an altered interaction with DNA (28). In earlier studies we described cell cycle dependent alterations of in vitro NHCP phosphorylation. Incubation of isolated NHCP's with [γ^{32}P]-

ATP has shown that NHCP's prepared from cells at various phases of the cell cycle showed alteration of ^{32}P incorporation (30).

It is an interesting question whether common chromatin changes are found after 1) cell cycle related alteration and 2) heat induced alteration of hyperthermic cell killing. In studies on fractionated hyperthermia, an intimate connection between formation of heat shock proteins and the development of thermotolerance, i.e. an induced resistance to otherwise lethal heat shocks, has been described (31-34). There is additional evidence that formation of the 68-70 kD heat shock proteins is altered after stimulation of cell proliferation (35).

In the present study we determined the period of lowest and highest thermosensitivity after stimulation of quiescent Reuber H35 cells to proliferation. We characterized the thermosensitive and thermoresistant cells by nuclear morphology and by the endogenous phosphorylation capacity of isolated NHCP's and we studied the effect of heat shock hereupon. Moreover we measured basal synthesis of HSP's in those cells. A relation between chromatin structure, endogenous NHCP phosphorylation capacity, expression of heat shock genes and thermosensitivity is discussed.

MATERIALS AND METHODS

Cells and culture conditions

The rat hepatoma cell line Reuber H35 was routinely grown at 37°C as monolayer cultures in 175 cm^2 plastic Greiner flasks. The medium consisted of Leibovitz-15 medium supplemented with 10 per cent foetal calf serum. Cells were synchronized by transferring them to serum-free medium for 96 hours. Afterwards the serum containing medium was added again at a time called for this purpose 'zero-time'. Cell cycle analysis by flow cytofluorometry was performed as described elsewhere (36).

Heat shock

For application of heat shock, culture flasks were immersed in a water bath. The heater was a circulating Thermomix 1420 (Braun. A.G. Melsungen, West Germany) provided with a proportional control of heating power giving stable temperature levels of ± 0.05°C (S.E.) within the range used. Temperature equilibration between the culture medium and the waterbath was obtained within 4 min.

Survival determination

The colony forming ability in vitro was used as a measure of survival. After treatment, cells were trypsinized, counted in a Coulter counter and plated in appropriate numbers into 25 cm^2 Greiner flasks. Seven days after plating, the cells were fixed and stained with Giemsa, and colonies were counted.

Nuclear texture

The Feulgen reaction not only depends on the actual amount of DNA present in the nucleus, but is also reflecting the state of the deoxy-nucleoprotein complex and the degree of chromatin condensation (37). For chromatin study in situ, cultures subconfluently grown in plastic Greiner flasks were fixed for 30 minutes in a mixture containing 85% methanol, 10% formalin and 5% glacial acetic acid. Hydrolysis was performed in 5N HCl at room temperature. The cells were stained with Schiffs reagent for 60 minutes in the dark (38). In order to avoid systematic differences due to reagents, cell fixatives and hydrolysis, the staining of duplicate specimen was conducted in parallel in the same reagents. The same reagent stocks were used for specimens sampled at other times. Feulgen-DNA measurements were done with a Zeiss scanning cytofluorometer 0.1. Each slide was used as its own blank to define 0.0 O.D. and densitometric calibration was checked by means of a neutral density filter.

Preparation of non-histone chromosomal proteins

Subconfluent monolayer cultures of H35 cells were washed twice with ice cold phosphate buffered saline (PBS), scraped in sucrose buffer (0.27 M sucrose, 0.1 mM EDTA, 5 mM Tricine-KOH pH 7.4) and centrifuged for 15 min. 500xg. Afterwards cells were suspended in 1 ml. sucrose buffer and homogenized by 10 strokes of a tight pestle Dounce homogenizer. After disruption of the cells KCl and $MgCl_2$ were added to a final concentration of 7.5 mM and 1 mM respectively. The homogenate was centrifuged for 15 min. 500xg. The sediment which contained the nuclei was washed with 75 mM NaCl containing 24 mM EDTA, pH 7.0. Chromatin was isolated by suspending the nuclei in 2 ml. of 0.85 M sucrose followed by centrifugation for 30 min. at 10.000xg. The sediment was washed with the NaCl-EDTA solution and extracted overnight with 1 ml. of 1 M NaCl. It was then centrifuged for 30 min. at 10.000xg. Nucleohistones were precipitated by the addition of 1.5 volume of destilled water and removed by 60 min. at 10.000xg. The supernatant, which contained NHCP's, was dialyzed over-

night against 0.4 M NaCl, 50 mM Tris buffer (pH 7.8). NHCP's were concentrated by use of a centricon-30 system (Amicon Corporation, Danvers).

Phosphorylation of non-histone chromosomal proteins

Non-histone chromosomal proteins incorporate ^{32}P from [γ -^{32}P] ATP indicating that they contain protein kinase activity and the appropriate substrates (30). Samples containing 200 ug of NHCP's (protein concentration was determined according to Lowry (39) as modified by Peterson (40)) were incubated for 1 h. at 37°C in the presence of 80 umole [γ-^{32}P] ATP containing approximately 8 x 10^6 cpm, 10 mM $MgCl_2$, 0.1 M Tris buffer (pH 7.5) in a total of 0.25 ml. For measurement of "total" ^{32}P incorporation the reaction was stopped by addition of 200 ug of non-radioactive ATP followed by dialysis overnight against 10 M urea, 0.5 M β-mercaptoethanol, 0.9 M acetic acid. Samples were loaded on 10 x 0.6 cm disc gels and electroforeses were performed for 4.5 h. at 2 mA according to the technique of Panyim and Chalkley (41) as modified by Shaw and Huang (42). The gels were sliced and the radioactivity of each slide measured by the Cerenkov effect in an Intertechnique liquid scintillator SL36.

For qualitative analysis of ^{32}P-labeled proteins, by use of one-dimensional SDS-polyacrylamide gel electrophoresis according to Laemmli (43), NHCP's were precipitated by TCA (final concentration of 10%). Protein was centrifuged 5 min. 8000xg and the sediment was washed with diethyl-ether. Finally protein was solubilized in sample buffer (5% SDS, 10% β-mercaptoethanol, 0.0015% bromophenol blue, 15% glycerol and 125 mM Tris-HCl, pH 6.8). The samples were electrophorized (see section on 2D- electrophoresis) and exposed to X-ray film. For establishing molecular masses of phosphoproteins, molecular mass markers including phosphorylase (92.000), bovine serum albumine (67.500), ovalbumine (45.000), chymotrypsinogen (25.000) and cytochrome C (12.500) (Bio Rad) were used.

Labeling of proteins and 2D-polyacrylamide gel elctrophoresis

For electrophoresis of proteins, cells were incubated in a methionine-free medium supplemented with 100 uCi [^{35}S]-methionine (sp. act. 363 Ci/mmol)/ml of medium for 60 minutes. The monolayers were washed twice with buffered saline and scraped in lysis buffer containing 9.5 M urea, 2% nonidet, 2% ampholines (pH 5-8: pH 3.5-10 = 4:1) and 5% β-mercapto-ethanol.

For two-dimensional electrophoresis of proteins the technique of O'Farrell (44) was used. Proteins were separated on cylindrical gels by isoelectric focusing with 9 M urea, 4% acrylamide and 5% ampholines (pH 5-8: pH 3.5-10 = 4:1). The second dimension was a 10% acrylamide discontinuous SDS gel (pH 8.8) with a 5% stacker gel (pH 6.8). Samples containing about 1.5 . 10^6 cpm labeled protein were applied to each gel. Fluorographs were made up with En^3Hance. Gels were dried and exposed to X-ray film.

Quantitation of spots

Spots on X-ray film were analysed and quantified using a Quantimet 720 automated image analyser (35). The optical density of every 0.16 mm^2 in fluorographic film was measured and converted into cpm by using a standard curve of optical density versus radioactivity on the same film. Relative rates of synthesis were calculated by normalization which occurred by dividing the integrated density of the spot by the integrated density of the total amount of spots.

Materials

Foetal calf serum was obtained from Sera-lab (Sussex-England). Radioactive chemicals were from the Radiochemical Centre (Amersham, England). All tissue components were from Gibco Grand Island Biological Co. (Grand Island N.Y.). Ampholines were purchased from LKB (LKB Instruments Inc. Rockville M.D.), while En^3Hance was obtained from New England Nuclear.

RESULTS

Cell cycle dependence of heat induced cell killing

Reuber H35 cells were synchronized by incubating subconfluent cell cultures in serum-free L15 medium for 4 days. Growth was re-initiated by exchanging serum-free medium for fresh 10% foetal calf serum containing L15 medium. Figure 1 is a composite figure showing the cytofluorometric data of cultures at various times after serum stimulation (Fig. 1 upper pannel). Cells appear in S phase at 8 h. and division takes place between 12 and 16 h. Subsequently cells enter the S phase again at 20-24 h.

Cell cycle dependence of heat induced cell killing was determined by heating cells for 1 h. at 42.5°C at various times after readdition of fresh serum containing L15 medium (Fig. 1). Cell viability of serum-free Go cultures was 0.3% but raised 5 fold when fresh serum containing medium

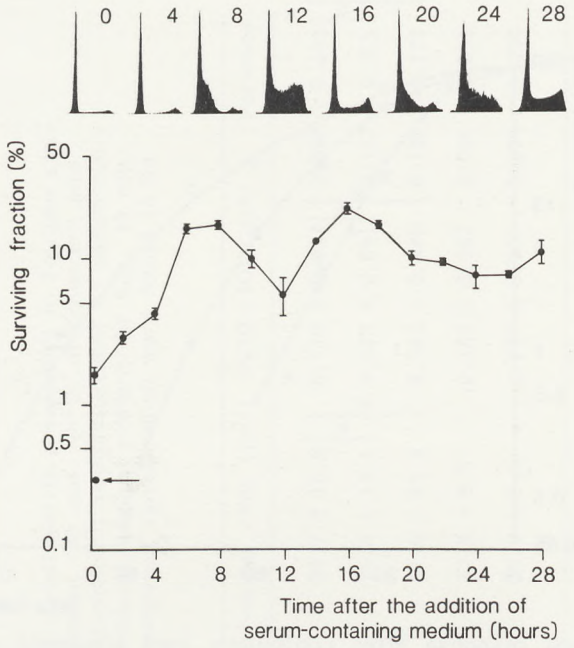

Fig. 1. Survival response after heat shock (1 h, 42,5°C) of quiescent Reuber H35 cells stimulated by serum containing medium for various time periods. Survival response of non-stimulated quiescent cells is indicated by arrow. Points, means of 3 determinations: bars, S.E.
Upper panel: Parallel cultures of cells were used for determination of cell cycle distribution by means of cytofluorometry. Cells were trypsinized and prepared for cytofluorometric analysis at times indicated.

had been present during the heat shock. Cell viability further increased as high as 16% when cells were heated during G1 or early S phase, and decreased again when a major portion of cells was in S and G2 phase. When cells entered G1 phase again, cell viability increased.

Alterations of heat sensitivity of Reuber H35 cells at various times during G1 phase was further characterised by assaying survival of cells exposed to 42.5°C for various lengths of time (Fig. 2). Viability of serum-free Go cells heated for 35 min. was 10%. When serum containing medium had been added for 2 or 8 hours, the heating time required to reduce cell survival to 10% increased to 55 and 77 min. respectively. With the data presented here, it is impossible to say whether the alterations in thermo-sensitivity is caused by modification of either survival curve shoulder or

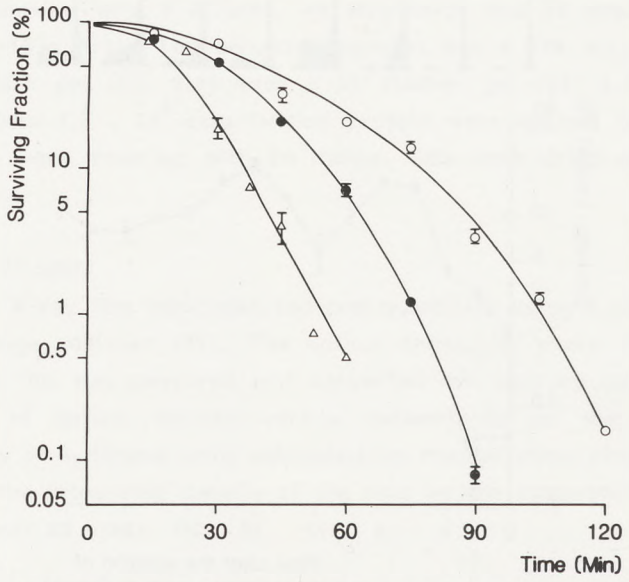

Fig. 2. Survival response after continuous heat treatment at 42,5°C for various lengths of time of quiescent Reuber H35 hepatoma cells (△) and cells stimulated by serum containing medium for 2 (●) and 8 hours (○) respectively. Points, means of 3 determinations: bars, S.E.

survival curve slope, because the curve does not become log-linear.

Influence of hyperthermia on nuclear texture in Go- and late G1/early S phase cells.

Reuber H35 hepatoma cells in culture show a polygonal cell shape. Cells are well spread on the surface and show many cellular contacts. H35 cells exhibit different morphological characteristics after hyperthermia (45). Together with the alteration of cell shape also the shape of the nucleus altered (15). To study the effect of heat treatment on nuclear shape and chromatin structuring of highly thermosensitive Go and thermoresistant late G1 cells, geometric and densitometric analysis of the nuclear projection area was performed. In order to avoid long treatment times and the occurrence of heat induced thermotolerance of morphological characteristics of the cell (45) we have chosen for a 30 min. 44°C treatment. The effect of this heat treatment on 1) average projection area, 2) integrated optical density (IOD) 3) average optical density (AOD) and 4) the variance in

Table 1 Measured and computed mean values of geometric/densitometric parameters in Feulgen stained samples prepared before and after heat treatment (30 min. 44°C) of quiescent and serum stimulated Reuber H35 cells: a) no serum containing medium present b) serum containing medium present for 30 min. c) serum containing medium present for 6 h. 30 min. Values were tested by Student's-test. (*): compared values which were found to be significantly different ($p < 0.05$).

	Temp (°C)	IOD	Nuclear Area (μm^2)	AOD (IOD/μm^2)	Variance
a) Go (-serum)	37	6.67 ± 1.01	39.7 ± 10.6 *	0.175 ± 0.043 *	0.087 ± 0.042 *
	44 (t=0-t=0.30)	7.35 ± 1.33	29.4 ± 10.1 *	0.278 ± 0.091 *	0.157 ± 0.193 *
b) Go (+serum)	44 (t=0-t=0.30)	7.44 ± 1.62	31.0 ± 10.3	0.262 ± 0.088	0.188 ± 0.179
c) G1/early S	37	6.11 ± 1.57	37.5 ± 9.5	0.180 ± 0.063	0.081 ± 0.035
	44 (t=6-t=6.30)	6.73 ± 1.26	36.0 ± 9.9	0.197 ± 0.057	0.094 ± 0.094

optical density per unit nuclear area, is shown in Table 1. Geometric image analysis before and after heat treatment was done on Feulgen stained nuclei of Go cells and they showed 26% reduction of area after heat treatment, indicating rounding-up of the cell nucleus. A similar effect was observed when fresh serum containing medium was present during heat treatment of Go cells. The same treatment had no effect on nuclear projection area of cells which had been stimulated by fresh serum containing medium for 6 hours.

To study heat induced alterations of chromatin structuring, densitometric analysis of Feulgen stained nuclei before and after heat treatment was performed by measuring optical density values of the nucleus and computing the IOD and the variance in OD per unit nuclear area. The average optical density was obtained by dividing IOD by area. As shown by Table 1, the AOD as well as the variance in Feulgen staining of Go cell nuclei was increased significantly ($p < 0.05$) after heat treatment, irrespective the presence of fresh serum containing medium during treatment. The IOD value remained significantly the same under these conditions. However, heat treatment did not influence IOD, AOD or variance when cells were stimulated by serum medium for 6 hours.

Although stimulation with fresh serum containing medium had no immediate protective effect on heat induced (30 min.44°C) alteration of nuclear morphology in Go cells, it still remained possible that protection by serum containing medium could be measured when applicating a lower heat dose. Fresh serum containing medium added just before a 30 minutes heat treatment at 43°C indeed protected against heat induced decrease of nuclear projection area (indicating protection against heat induced rounding-up). Nuclear projection area decreased 18 per cent in cells treated under serum-free conditions and 5 per cent when fresh serum containing medium was added to the cells just before heat treatment (data not shown).

It can be concluded that in contrast to Go cell nuclei, the nuclei of late G1/early S cells were relatively thermoresistant. A slight increase of thermoresistance was found immediately upon stimulation of Go cells with serum containing medium.

Effect of heat treatment of Go and late G1/early S phase cells on endogenous phosphorylation capacity of isolated non-histone chromosomal proteins

The alteration of thermosensitivity during progression of cells from Go

Table 2 Effect of heat treatment on ^{32}P incorporation into NHCP's which were isolated from Go phase or G1/early S phase cells, before or after heat treatment (30 min. 42.7°C). Samples containing 200 ug of protein were incubated with radioactive ATP for 1 h at 37°C and analysed by electrophoresis on polyacrylamide gel according to Panyim and Chalkley (41) modified by Shaw and Huang (42) followed by gel slicing and radioactivity measurement.

		cpm x 10^{-3}/200 ug NHCP's
Go	37°C	352
	42.7°C 30 min.	933
G1/early S	37°C	690
	42.7°C 30 min.	878

into S phase, as it appeared from monitoring the fraction of surviving cells and from the description of nuclear/chromatin structure is evident, but the molecular basis is yet unclear. In order to approach this, we asked the question whether also certain molecular processes show this altered thermosensitivity and focused our attention on NHCP's.

NHCP's contain protein kinases and phosphoproteins: when NHCP's are isolated and subsequently incubated with [γ-^{32}P] ATP, radioactive phosphate is incorporated in protein. Chromatin prepared from Reuber H35 cells and incubated with [γ-^{32}P] ATP showed increased incorporation of radioactive phosphate when 1) resting cells were stimulated to proliferation by serum containing medium (30) and 2) exponentially growing cells were heat treated (12).

This raised the possibility to test the effect of heat treatment on chromatin of thermosensitive Go cells and thermoresistant late G1/early S cells by measuring the in vitro phosphorylation of NHCP's isolated from these cells: ^{32}P incorporated into NHCP's was measured as described before (12,30) by gel electrophoresis according to the technique of Panyim and Chalkley (41) as modified by Shaw and Huang (42) followed by gel slicing and radioactivity measurement. Moreover, samples were prepared for gel electrophoresis according to the procedure of Laemmli (43) followed by autoradiography.

Analysis of ^{32}P incorporation into NHCP's isolated before and after heat treatment (30 min. 42.7°C) was performed for Go and late G1/early S phase cells. When Go cells were heat treated and NHCP's isolated and 'in

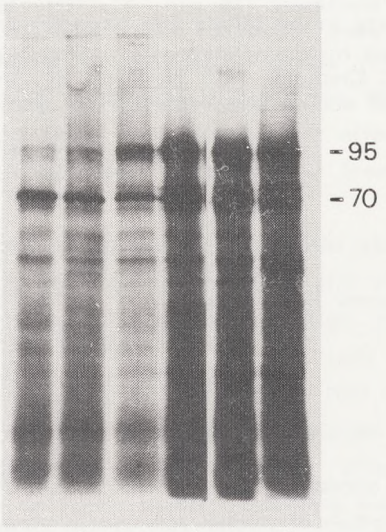

Fig. 3. Phosphorylated proteins after in vitro phosphorylation of NHCP's isolated from cells after stimulation with fresh serum containing medium and/or hyperthermic treatment.
NHCP's were prepared from Go cells (a,d) or, from cells which were stimulated with fresh serum containing medium for 30 min. (b,e) or from late G1/early S phase cells after growth stimulation for 7 hours (c,f). The NHCP's were isolated either from non-heated cells (a,b,c) or from heated cells (30 min. 42.7°C) immediately after heat treatment (d,e,f). Samples containing 200 ug of protein were incubated with radioactive ATP for 1 h. at 37°C and analysed by polyacrylamide gel electrophoresis performed according to Laemmli (43).

vitro' phosphorylated, the ^{32}P incorporation increased with 165%, while increase of ^{32}P incorporation was 27% for heated late G1/early S phase cells (Table 2). Probably, late G1/early S cells were thermoresistant as compared to Go cells, as shown by the ability of endogenous phosphorylation of isolated NHCP's. Besides alterations of 'over-all' phosphorylation capacity by serum stimulation of resting Reuber H35 cells and by heat treatment, also phosphorylation of individual NHCP's was altered (Fig. 3). For Go cells, most of the ^{32}P was incorporated by protein of Mr 70.000. In cells stimulated by fresh serum containing medium for 0.5 h. the relative intensity of a band at Mr 95.000 increased as compared to the band at Mr 70.000. This effect was enhanced after 7 hours growth stimulation, in late G1/early S phase cells.

After heat treatment the in vitro phosphorylation pattern showed an enhancement of incorporation by protein of Mr 95.000, the enhancement was more for Go cells as compared to cells stimulated with serum containing medium. The data indicate that the major qualitative changes in endogenous phosphorylation of NHCP's caused by serum stimulation as well as by heat shock is the enhanced ^{32}P incorporation of protein with molecular weight of Mr 95.000.

Basal synthesis of HSP's in Go and in late G1/early S phase cells

One of the most extensively described phenomena induced by heat shock in mammalian cells is an alteration of gene expression, resulting in heat shock protein synthesis. In studies on fractionated hyperthermia, an intimate connection between formation of HSP's and the development of thermotolerance, i.e. an induced resistance to otherwise lethal heat shocks, has been described (31-34). It has also been suggested that heat sensitivity of cells that were not heated before could be related to basal HSP synthesis level (46,47). The question raised whether the alteration of thermal resistance of the cell during the prereplicative period could be related to HSP synthesis.

For the identification of HSP's, exponentially growing cells were heated (45 min. 42°C) and proteins were separated by two-dimensional gel electrophoresis (Fig. 4A,B,C). For an exact localization of HSP 68 on autoradiograph a 2D gel from cells treated with lower heat dose (20 min. 42°C) was used (autoradiograph not shown). Five HSP's, indicated as HSP 65, HSP 68, HSP 70, HSP 84 and HSP 100 have been used in our further studies. The synthesis rates of these proteins were determined in synchronized H35 cells by incubation with [^{35}S] methionine for 1 hour of cells during Go, early G1, late G1/early S, mid S and late S/G2 phase (indications of cell cycle phase was based on cytofluorometric data, see Fig. 1). In order to study basal non-induced HSP synthesis, HSP 68 included, it was necessary to view longer exposures. Protein synthesis patterns of Go, late G1/early S and late S/G2 phase cells are shown in Fig. 4D,E,F. Percent of radioactivity incorporated in HSP during 1 h. of synthesis at various cell cycle stages is shown in Tabel 3. Synthesis of HSP 65, HSP 68 and HSP 84 (HSP 84 was found as a composite spot, consisting of overlapping smaller spots) was clearly enhanced when Go cells were stimulated to proliferation by addition of fresh serum containing medium. Relative synthesis rates of those proteins was at a maximum during late G1/early S phase and decreased when cells progressed throughout S phase. In contrast, hardly

Table 3 Relative rate of individual HSP synthesis of synchronized Reuber H35 cells as the population progressed through the cell cycle. Cells were pulse-labeled with [^{35}S]-methionine during Go, early G1, G1/early S, mid S or late S/G2 (indication of cell cycle phase was based on cytofluorometric data as shown in Fig. 1) for 1 hour. The synthetizing rate of individual HSP's was obtained by densitometry of autoradiographs from 2D-gels followed by conversion into radioactivity (see section on Materials and Methods). Each value listed below indicates synthesis rate of individual HSP's as percentage of total protein synthesis rate. Data for samples indicated Go, G1/early S and late S/G2 were derived from the autoradiographs shown in Fig. 3D, 3E and 3F, respectively.

	HSP 65	HSP 68	HSP 70	HSP 84	HSP 100
Go	0.46	0.00018	0.57	0.23	0.27
early G1	0.84	0.00022	0.65	0.42	0.32
G1/early S	1.85	0.00140	0.74	0.57	0.33
mid S	0.79	0.00110	0.65	0.30	0.34
late S/G2	1.03	0.00050	0.74	0.25	0.26

any change in relative synthesis of HSP 70 and HSP 100 was found throughout the cell cycle.

Apparently, alterations of thermoresistance upon stimulation to proliferation of resting H35 cells runs parallel with increased levels of basal synthesis of HSP 65, HSP 68 and HSP 84. However, the enhancement of HSP synthesis upon growth stimulation is small as compared to enhancement of HSP synthesis induced by heat shock.

DISCUSSION

The results presented in this paper show that thermoresistance alters during the prereplicative period. It increased after addition of serum containing medium to quiescent cultures and maximal thermal resistance was reached in late G1 phase.

Variations in thermal sensitivity during the cell cycle have been established since Giovanella et al. (1) used synchronized cultures to study the cell cycle phase dependent hyperthermic killing (2-4). It has been generally found that late S phase cells are the most sensitive to hyperthermic treatment. In contrast, lowest sensitivity was observed in cells near the G1/S boundary. These results agree quite well with our data

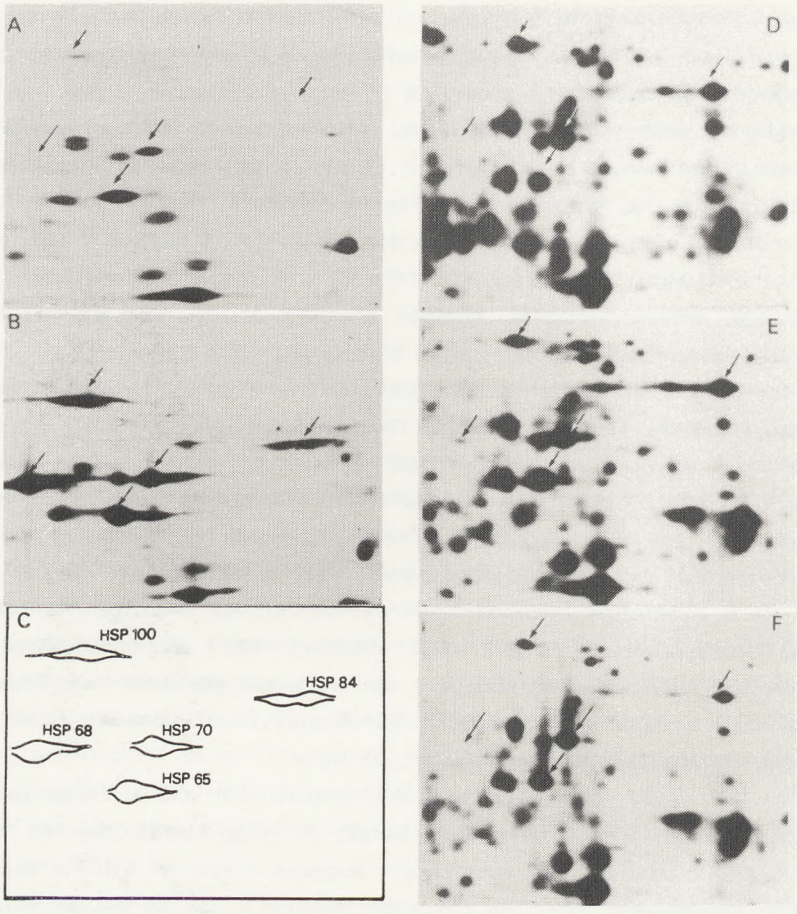

Fig. 4. Synthesis of HSP's in quiescent Reuber H35 hepatoma cells and in cells stimulated by serum containing medium as analysed by 2D-gel electrophoresis followed by autoradiography.

A/B/C: For localizing HSP's, parallel cultures of exponential growing H35 cells were incubated at 37°C with [^{35}S]-methionine for 1 h (A) or heated at 42°C for 45 min. and subsequently reincubated at 37°C with [^{35}S]-methionine for 1 h (B). Localizations of HSP's are indicated in (C).

D/E/F: Quiescent H35 cells (D) and serum-stimulated cells during late G1/early S (E) and late S/G2 phase (F) of the cell cycle were incubated with [^{35}S]-methionine for 1 hour (cell cycle phases were established by cytofluorometry as shown in Fig. 1). Each gel was prepared for fluorography and exposed to film for autoradiography. The arrows point to the localization of HSP 65, HSP 68, HSP 70, HSP 84 and HSP 100 as has been established in (B). The synthesis rate of individual HSP's was calculated and values are shown by Table 3.

which were obtained with cells synchronized by serum deprivation. Furthermore our data point to an increased sensitivity of cells in their quiescent state.

As shown in Fig. 1, addition of serum containing medium to Go cells resulted in an immediate expression of thermal resistance. Cell viability of Go cells increased 5 fold when fresh serum containing medium was added to the cells just before heat treatment. Cell viability further increased when cells were heated during G1 or early S phase. This increase in heat resistance as monitored by the fraction of surviving cells was expressed in two other ways: 1) tolerance of the nuclear shape and chromatin structuring as characterized by Feulgen staining, 2) the capacity of isolated non-histone chromosomal protein to be phosphorylated in vitro.

Normally when studying thermosensitivity, survival is used as endpoint of observation. In studies on fractionated hyperthermia an induced resistance to otherwise lethal heat shocks (thermotolerance) has been observed which runs parallel with an increased resistance of cell morphology (45,48), cytoskeleton (49), protein synthesis (8,45), DNA synthesis (8) and nuclear morphology (15). Whether there is also during cell cycle a relation between hyperthermic killing and heat resistance of those processes c.q. structures is unknown. Here we demonstrate in the comparison between heat sensitive Go and heat resistant late G1/early S cells such a relation for nuclear morphology, chromatin structuring and endogenous NHCP phosphorylation capacity.

In this study we focused special attention on a role of chromatin structuring and NHCP's in thermoresistance. Although untill now we can not prove any causality, the data shown suggest a role of NHCP's in regulation of thermosensitivity. We compared features of phosphorylation capacity of NHCP's isolated from thermoresistant late G1/early S cells with those of thermosensitive Go cells: 1) The in vitro phosphorylation capacity was two times more in NHCP's isolated from thermoresistant late G1/early S cells. 2) For late G1/early S phase cells ^{32}P incorporation in protein with molecular weight Mr 95.000 was enhanced as compared to Go phase cells. Interestingly, heat treatment of Reuber H35 cells resulted also in an increase of in vitro phosphorylation capacity while a major increase of phosphorylation also was found on NHCP with molecular weight of 95 kD. Possibly, those 'over-all' and qualitative alterations in NHCP phosphorylation reflect 'in vivo' alterations in NHCP's which are required for development of thermoresistance upon stimulation with serum containing medium of resting Reuber H35 cells as well as for development of so called

'acquired thermotolerance' after heat treatment.

At the moment we do not know what the underlying mechanism is for alteration of endogenous phosphorylation capacity of isolated NHCP's. The change in the extent of NHCP phosphorylation in vitro may be interpreted in several ways (50): a) Changes in molecular species of the endogenous substrates. b) Changes in protein conformation which could change the number of phosphorylating sites. c) Changes in the number of sites of protein phosphorylation available in vitro. d) Increase in protein kinases specific for endogenous substrates. e) Alteration of the specific activity of protein kinases. These possibilities are under present investigation.

It has been suggested that an induced transient reprogramming of cellular activities protects the system from irreversible stress injury and allows a rapid and complete resumption of normal cellular activities after the stress period (57). In our opinion NHCP's will be a serious candidate to participate in reprogramming those cellular activities leading to alteration of cell cycling and alteration of thermosensitivity both after stimulation of resting cells and after heat treatment.

One of the most extensively studied responses in heat treated cells is the synthesis of heat shock proteins (for reviews see 51-54). A considerable number of facts suggest an essential function of HSP's in protecting cells against heat damage. Less data are available on the regulation of basal HSP synthesis (55,56,57). In this study we demonstrate that during the thermoresistant late G1/early S phase there is an enhanced basal synthesis of HSP 65, HSP 68 and HSP 84. These and other data (58-62) suggest that increase of HSP synthesis is connected with increase of DNA replication.

A number of cellular characteristics are different in late G1/early S phase cells as compared to Go cells. This paper gives an inventory of a few of them. It seems interesting to look for causal relationships. The most obvious relationship might be: alteration of chromatin structure (reflected in the alteration of NHCP's capacity to become phosphorylated after isolation) leads to alteration of gene expression e.g. HSP synthesis. But HSP-mRNA synthesis still has to be measured under these experimental conditions. Preliminary results in our laboratory indicate that alteration of endogenous NHCP phosphorylation capacity is an early event in heat shock response. We measured enhanced phosphorylation of protein Mr. 70.000 in NHCP's isolated from cells heated for 5 minutes at 42.7°C (as in thermosensitive Go cells) while phosphorylation of protein Mr. 95.000 was relatively increased when the treatment was extended (as in thermoresistent

G1/early S phase cells).

Resting Reuber H35 cells stimulated to proliferation seems a promising system to give insight in the molecular events at chromatin level leading to 1) alteration of thermosensitivity without alteration of HSP synthesis (63-67) and 2) alterations of thermosensitivity connected with alteration of HSP synthesis (31-34).

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Chapter 8

Heat-induced alterations in non-histone chromosomal proteins, chromatin associated heat shock protein and the occurrence of thermotolerance in Reuber H35 hepatoma cells

(Submitted for Int. J. Hyperthermia)

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SUMMARY

Heat treatment of Reuber H35 rat hepatoma cells at 43°C for 30 min. induced both, alterations in molecular properties of chromatin and expression of thermotolerance. Chromatin was altered as was shown by alterations in endogenous phosphorylation of isolated non-histone chromosomal proteins (NHCP's). Moreover, by use of cell fractionation, it was demonstrated that newly synthesized heat shock proteins with molecular weights ranging from 68.000-70.000 dalton (HSP 68-70) became attached with the chromatin fraction (and nuclear matrix). For getting insight in the sequence of heat induced events, kinetics of (a) alterations in NHCP's, (b) synthesis of HSP's and their association with chromatin, and (c) expression of thermotolerance, were compared.

Within 10 minutes after a shift to 43°C of Reuber H35 cells changes in chromatin were demonstrated. These alterations were reflected in an increase of endogenous phosphorylation capacity of isolated NHCP's. Most ^{32}P was incorporated by proteins of Mr 70.000 and Mr 95.000. For cells that had been heated for 30 minutes, phosphorylation was further changed and most ^{32}P was incorporated by protein of Mr 95.000. Initiation of these

heat induced alterations of chromatin preceded the increase of HSP synthesis. Enhancement of HSP synthesis was observed between 0.5 and 1.5 hours after heat treatment upon reincubation at 37°C while in parallel the level of newly synthesized HSP 68-70 associated with chromatin and nuclear matrix increased. Within the next 6 hours of recovery a further extension of the level of HSP 68-70 attached to chromatin and nuclear matrix was observed followed by diminishing of the level. During this time period thermotolerance fully developed and it remained expressed for at least the next 18 hours.

A role for NHCP's and HSP's in regulation of thermosensitivity after heat shock has been discussed.

INTRODUCTION

Mammalian cells become rapidly inactivated following elevation of temperature above 42°C. The hyperthermic response is however influenced by a number of parameters such as nutritional conditions (1-3), pH (4), oxygen (5), cell cycle phase (6,7) and thermal history of the cell (7-9). An interesting aspect of thermal biology is the response of heated cells to a subsequent heat challenge: pretreatment with a heat shock improves the ability of surviving cells to withstand a second heat shock (8,9). Neither the mechanism of heat induced cell killing nor the activities which protect the cell from irreversible stress are well understood. However there is evidence that damage within the chromatin is responsible for cell lethality (10).

In a recent study we considered regulation of thermosensitivity at chromatin level during cell cycle (11). After growth stimulation of resting Reuber H35 cells, cellular thermoresistance increased in parallel with the thermoresistance of structural and molecular properties of the chromatin. Furthermore, upon growth stimulation, alterations in NHCP's seem to precede enhancement of basal HSP synthesis.

Heat shock proteins might be essential for protection against heat damage but also for a rapid and complete recovery of cellular activities after treatment (12). However no specific functions of HSP's are known yet. An approach which has been used in searching for the function of these proteins is to study their location within the cell by use of specific antibodies or by cell fractionation. In studies on the cellular distribution of HSP's, HSP 68 and HSP 70 were located in the chromatin fraction sometimes attached to the nuclear matrix (13-19). Such data are evidence for a

nuclear function of these HSP's.

Since these studies deal with either the altered molecular properties of chromatin or with altered cellular distribution of HSP's, it is not clear whether these alterations are related to each other and to cellular thermosensitivity. In our approach we studied Reuber H35 hepatoma cells after a single treatment and described (a) alterations in chromatin as reflected by the alterations of endogenous phosphorylation capacity of isolated NHCP's (b) synthesis of HSP's and their association with chromatin and nuclear matrix and (c) development of thermotolerance.

It is concluded that a heat treatment for 30 min. at 43°C induces alterations of chromatin which precede the synthesis of HSP's. In parallel with accumulation of HSP 68-70 in the chromatin fraction, an increase of thermotolerance was found. These heat induced alterations in chromatin diminished concomitantly, but a long time before decay of cellular thermotolerance.

MATERIALS AND METHODS

Cell culture and radiolabeling

Rat Reuber H35 cells were routinely grown at 37°C as monolayer cultures in plastic tissue culture flasks (Greiner). The medium consisted of Leibovitz-15 medium supplemented with 10% foetal calf serum. For electrophoresis of proteins, cells were incubated in a methionine-free medium supplemented with 8 uCi [^{35}S]-methionine (sp. act. 363 Ci/mmol)/ml.

Heat shock

For applicating a heat shock, culture flasks were immersed in a water bath. The heater was a circulating Thermomix 1420 (Braun A.G. Melsungen, West Germany) provided with a proportional control of heating power giving stable temperature levels of ± 0.05°C (S.E.) within the range used. Temperature equilibration between culture medium and the water bath was obtained within 5 minutes.

Survival determination

The colony forming ability in vitro was used as a measure of survival. After treatment, cells were trypsinized, counted and plated in appropriate numbers into 25 cm^2 Greiner flasks. Seven days after plating, the cells were fixed and stained with Giemsa, and colonies were counted.

Cell fractionation

Subconfluent monolayer cultures of H35 cells were washed twice with NKM (130 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$) scraped in NKM and centrifuged for 15 min 500xg. All operations were performed at 4°C in the presence of 0.1 mM phenylmethansulfonyl fluoride and 0.1 mM thiodiethanol. The cell pellet was resuspended in reticulocyte saline buffer (RSB: 10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.4) containing 1% Triton X-100 and centrifuged for 15 min. 500xg. The supernatant (indicated supernatant A) containing cytoplasmic materials and soluble or solubilized nuclear proteins was saved. The nuclei were resuspended in RSB containing 0.5% Na-deoxycholate and 0.5% Nonidet-P40 and centrifuged for 15 min. 500xg. The supernatant was combined with supernatant A and this formed the fraction containing cytoplasmic and free nuclear proteins.

For preparing chromatin, purified nuclei were washed with 75 mM NaCl containing 24 mM EDTA, pH 7.0. Chromatin was isolated by suspending the pellet in 2 ml. of 0.85 M sucrose followed by centrifugation at 10.000xg for 30 min.

For isolating nuclear matrices, purified nuclei were digested 15 min. at 20°C with bovine pancreas DNase I (50 ug/ml) and bovine pancreas RNase A (100 ug/ml). The digest was transferred to a tube filled with 1 ml. of 2.6 M sucrose (underlayer) and 9 ml. of 1 M sucrose in RSB and centrifuged for 15 min. in a Beckman SW41 rotor. The DNA/RNA depleted nuclei, concentrated on the heavy sucrose cushion, were gently resuspended in 5 ml. of 0.4 M $(NH_4)_2SO_4$, 30 mM Tris (pH 7.4), 10 mM $MgCl_2$ and centrifuged for 5 min. at 800xg. Nuclear matrices were resuspended in RSB. By this procedure four samples were obtained: 1) complete cells 2) fraction containing cytoplasmic material and soluble and solubilized nuclear material 3) chromatin and 4) nuclear matrices.

Preparation of non-histone chromosomal proteins

Subconfluent monolayer cultures of H35 cells were washed twice with ice cold phosphate buffered saline (PBS), scraped in sucrose buffer (0.27 M sucrose, 0.1 mM EDTA, 5 mM Tricine-KOH pH 7.4) and centrifuged for 15 min. 500xg. Afterwards cells were suspended in 1 ml. sucrose buffer and homogenized by 10 strokes of a tight pestle Dounce homogenizer. After disruption of the cells KCl and $MgCl_2$ were added to a final concentration of 7.5 mM and 1 mM respectively. The homogenate was centrifuged for 15 min. 500xg. The sediment which contained the nuclei was washed with 75 mM NaCl containing 24 mM EDTA, pH 7.0. Chromatin was isolated by sus-

pending the nuclei in 2 ml. 0.85 M sucrose followed by centrifugation at 10.000xg for 30 min. The sediment was washed with the NaCl-EDTA solution and extracted overnight with 1 ml. of 1 M NaCl. It was then centrifuged for 30 min. at 10.000xg. Nucleohistones were precipitated by the addition of 1.5 volume of distilled water and removed by 60 min. at 10.000xg. The supernatant which contained NHCP's was dialyzed overnight against 0.4 M NaCl, 50 mM Tris buffer (pH 7.8). NHCP's were concentrated by use of a centricon-30 system (Amicon Corporation, Danvers).

Phosphorylation of non-histone chromosomal proteins

Non-histone chromosomal proteins incorporate ^{32}P from [γ-^{32}P]-ATP indicating that they contain protein kinase activity and the appropriate substrates (20). Samples containing 200 ug of NCHP's (protein concentration was determined according to Lowry (21) as modified by Peterson (22)) were incubated for 1 h. at 37°C in the presence of 80 umole [γ-^{32}P]-ATP containing approximately $8x10^6$ cpm, 10 mM $MgCl_2$, 0.1 M Tris buffer (pH 7.5) in a total of 0.25 ml. For qualitative analysis of proteins which incorporated ^{32}P, NHCP's were precipitated by 10% TCA and separated by one-dimensional SDS-polyacrylamide gel electrophoresis according to Laemmli (23).

Gel electrophoresis

Samples for SDS-gel electrophoresis were prepared from cellular fractions by precipitating the proteins with 10% TCA. The precipitates were pelleted by centrifugation, washed with diethyl-ether and dissolved in sample buffer (5% SDS, 10% β-mercaptoethanol, 0.0015% bromophenol blue, 15% glycerol and 125 mM Tris-HCl, pH 6.8). The samples were electrophorized on 10% polyacrylamide gels. For establishing molecular masses of proteins, molecular mass markers including phosphorylase (92.000), bovine serum albumine (67.500), ovalbumine (45.000), chymotrypsinogen (25.000) and cytochrome C (12.500) (BioRad) were used. For electrophorized samples containing [^{35}S]-methionine fluorographs were made up with En^{3}Hance. All gels were dried and exposed to X-ray film.

Materials

Foetal calf serum was obtained from Sera-lab (Sussex, England). Radioactive chemicals were from the Radiochemical Centre (Amersham, England). All tissue components were from Gibco Grand Island Biological Co. (Grand Island N.Y.). DNase I and RNase A were purchased from

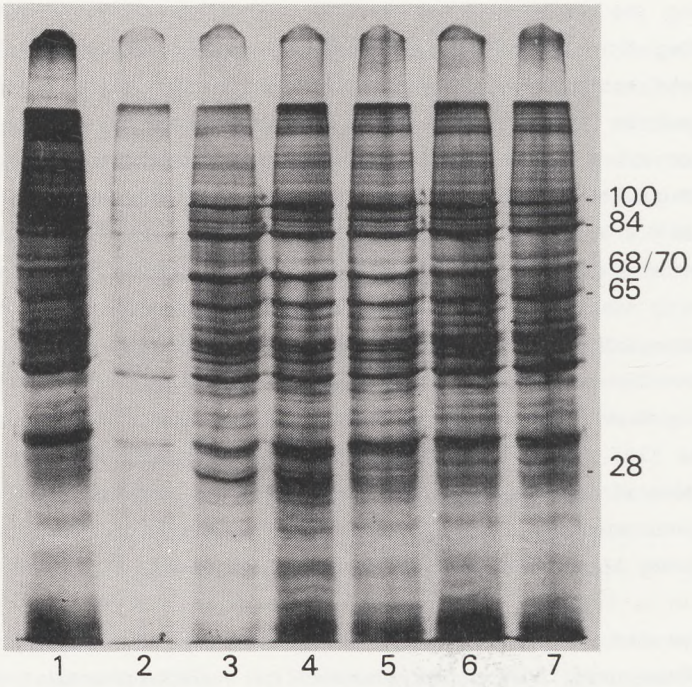

Fig. 1. Protein synthesis pattern of control and heat treated (30 min. 43°C) H35 hepatoma cells. Lane 1: proteins from control cells. Lane 2-7: Proteins from heat shocked cells. Pulse labeling for 1 hour with [^{35}S]-methionine started at 0, 2, 4, 6, 8 and 10 hours from the beginning of heat treatment, respectively.

Sigma, while En^{3}Hance was obtained from New England Nuclear.

RESULTS

Kinetics of synthesis and cellular distribution of HSP's

As described previously, heat shock of Reuber H35 cells at 43°C for 30 min. resulted in the synthesis of heat shock proteins indicated as HSP 28 (28kD), HSP 65, HSP 68, HSP 70, HSP 84 and HSP 100 (24). Here we studied the kinetics of synthesis of those proteins after a heat treatment of 30 min. at 43°C by pulse-labeling proteins with [^{35}S]-methionine (Fig. 1). No enhanced incorporation was found at any of the bands till 30 minutes after heat treatment. Then the relative synthesis of HSP's increased during the next 2 hours. Synthesis of HSP 28 and HSP 68-70 remained elevated for about 4 and 6 hours respectively while synthesis of

HSP 65, HSP 84 and HSP 100 was enhanced for at least 10 hours. HSP 68 and HSP 70 were, in general, insufficiently separated by gel electrophoresis to allow analysis of each of the proteins. Therefore these proteins will be referred to as HSP 68-70. In these experiments, protein synthesis rate decreased during treatment to 20% of control value, and recovered upon reincubation at 37°C in about 6 hours (data not shown).

The distribution of heat shock proteins was studied by fractionation of cells in (1) cytoplasmic- and free nuclear proteins, (2) chromatin and (3) nuclear matrix. For this purpose cells were continuously labeled by incorporation in [^{35}S]-methionine containing medium, starting immediately after heat shock (30 min. 43°C), for various time periods. As shown in Fig. 2 the intracellular distribution of HSP 68-70 fluctuated. Accumulation of HSP 68-70 was found in total cellular protein (Fig. 2A) and in the fraction containing cytoplasm and free nuclear proteins (Fig. 2B). These levels remained high in the time period between 4 and 8 hours after heat shock. Furthermore the heat induced HSP 68-70 was found chromatin (Fig. 2C and 2E) and nuclear matrix (Fig. 2D and 2F) associated. These proteins accumulated in chromatin during the first 4 fours after heat treatment in parallel with their cellular accumulation. However during the next 4 hours the level of chromatin associated HSP 68-70 sharply declined. Similar kinetics were found for the association of HSP 68-70 with the nuclear matrix fraction. These data indicate that chromatin (and nuclear matrix) bound HSP 68-70 are specifically released from the chromatin and nuclear matrix fraction of the cell, 4 hours after heat treatment.

Kinetics of heat induced chromatin alterations as studied by endogenous phosphorylation of isolated non-histone chromosomal proteins

Hyperthermic treatment of Reuber H35 cells resulted in alterations of nuclear morphology and of the in vitro phosphorylation of NHCP's (25). NHCP's contain protein kinases and phosphoproteins: when NHCP's are isolated and subsequently incubated with [γ-^{32}P]-ATP, radioactive phosphate is incorporated into protein. Alterations in NHCP's induced in cultured cells might be detected in this endogenous NHCP phosphorylation assay. But any change in the extent of NHCP phosphorylation in vitro can be a result of changes in the substrates, kinases, phosphatases or the number of phosphorylating sites (26).

Here we describe alterations in NHCP's, as monitored by in vitro phosphorylation, during and after heat shock (30 min. 43°C). Endogenous NHCP phosphorylation capacity altered during a heat treatment at 43°C

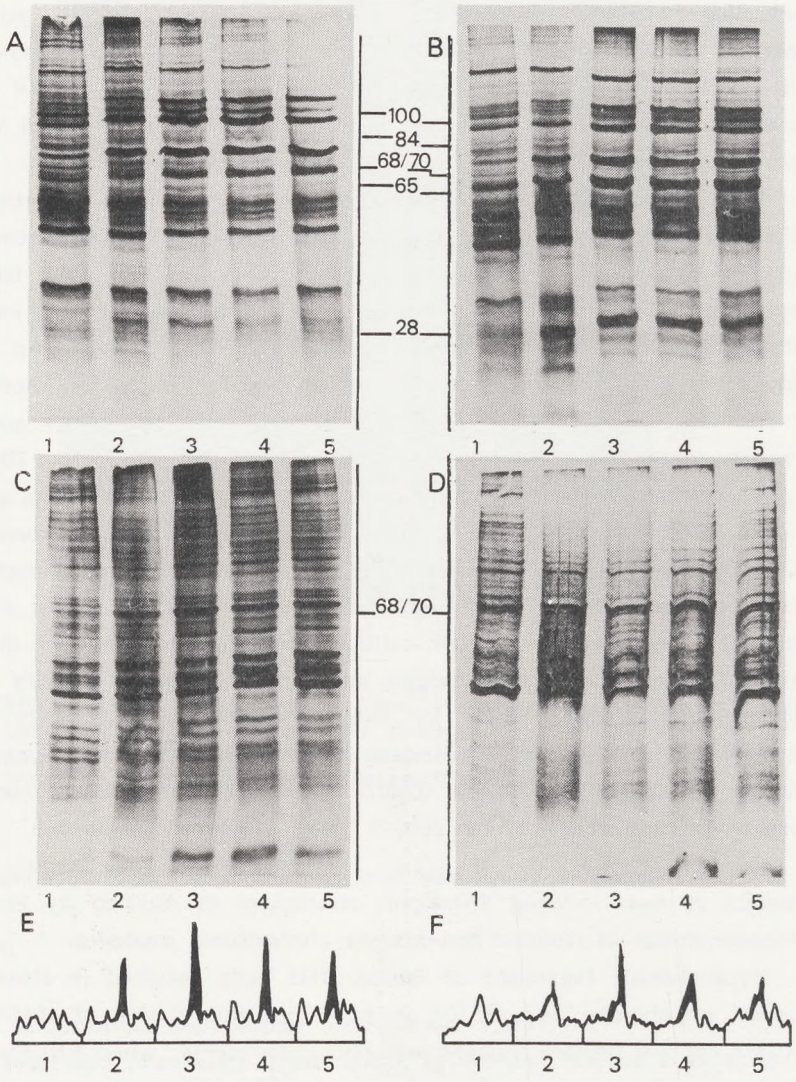

Fig. 2. Distribution of newly synthesized proteins among various subcellular fractions after heat shock (30 min. 43°C) of H35 hepatoma cells. Control cells (lane 1) were labeled with [^{35}S]-methionine for 4 hours while heated cells (lane 2-5) were labeled for 2, 4, 6 or 8 hours respectively, from the beginning of heat treatment. Afterwards cells were fractionated as described in the section on Materials and Methods. A) proteins from total cell extract. B) cytoplasmic and free nuclear proteins. C) chromatin fraction and D) nuclear matrix fraction. E) and F) are densitometric scans of the HSP 68-70 band from the fluorograms as shown in C and D respectively. Increase of optical density as a result of heat treatment has been indicated.

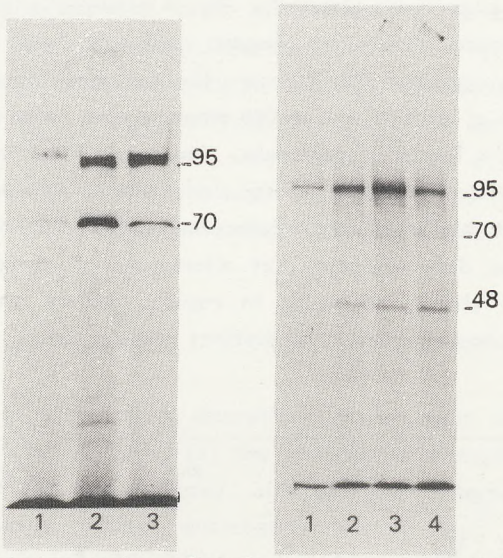

Fig. 3. Phosphorylated proteins after in vitro phosphorylation of NHCP's isolated from control cells and from cells heated for various times at 43°C. NHCP's were isolated from control cells (lane 1) from cells heated for 10 min. at 43°C (lane 2) and from cells heated for 30 min. at 43°C (lane 3). Samples containing 200 ug of protein were incubated with radioactive ATP for 1 hour at 37°C and analyzed by polyacrylamide gel electrophoresis.

Fig. 4. Phosphorylated proteins after in vitro phosphorylation of NHCP's isolated from control cells or from heated (30 min. 43°C) cells which were reincubated after treatment at 37°C for various times. NHCP's were prepared from control cells (lane 1), from cells heated for 30 min. at 43°C (lane 2) or from heated cells (30 min. 43°C) which were reincubated after treatment at 37°C for 3 hours (lane 3) or 6 hours (lane 4). Samples containing 200 ug of protein were incubated with radioactive ATP for 1 hour at 37°C and analyzed by polyacrylamide gel electrophoresis.

(Fig. 3 and 4). Several protein bands show major changes in phosphorylation, some of them early during heat treatment (Mr 70.000 and Mr 95.000) and others at later times (Mr 48.000). Although between experiments the time course of these changes can be different, e.g. the appearance of the Mr 48.000 band, some general conclusions could be drawn. In all experiments endogenous phosphorylation was already enhanced in NHCP's isolated from cells heated for 10 minutes. Most of the ^{32}P was incorporated by proteins of Mr 70.000 and Mr 95.000 (Fig. 3). However for cells that had been heated for 30 minutes, the endogenous phosphorylation of isolated NHCP's was further changed and most ^{32}P was incorporated by

protein of Mr 95.000.

In another experiment shown by Fig. 4 essentially the same differences between non-heated and 30 min. heated cells are shown concerning in vitro phosphorylation capacity. Furthermore it illustrates the increase of in vitro phosphorylation of protein Mr 48.000 when heated cells were reincubated at 37°C for 3 and 6 hours respectively. Moreover, phosphorylation of protein Mr 95.000 was further enhanced for cells which were reincubated at 37°C for 3 hours but decreased when reincubation time at 37°C was extended to 6 hours. These data indicate that alterations of chromatin occur during and after heat shock, resulting in rapid, mediate and slow (transient) effects in the phosphorylation of distinct protein bands.

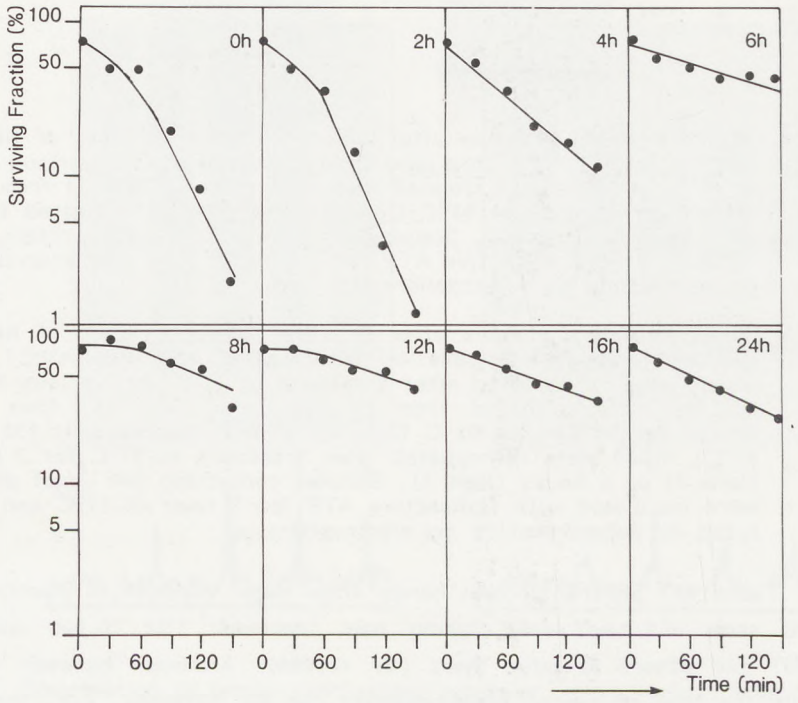

Fig. 5. Survival response of heat conditioned H35 cells. Survival curves at 43°C determined at various intervals at 37°C after heat conditioning at 43°C for 30 min. The fractionation intervals are indicated in hours. Points: mean of 3 determinations: Standard errors have been omitted but are less than 10%.

Expression of thermotolerance in Reuber H35 hepatoma cells

Reuber H35 cells exposed to a heat shock of 30 minutes at 43°C acquire resistance to a subsequent treatment at 43°C. Fig. 5 demonstrates the fraction of survival as function of the 37°C incubation time between initial and second heat treatment. Tolerance starts to develop between 2 and 4 hours after the initial heat shock and was at a maximum at 6-8 hours. Thermotolerance remained at least for the next 18 hours although a slight decay of tolerance was found after 12 hours from the initial heat treatment.

DISCUSSION

In this paper we demonstrate alterations of chromatin during and after a heat shock of 30 min. at 43°C: (a) the endogenous phosphorylation capacity of isolated NHCP's was changed, and (b) the pattern of newly synthesized proteins was specifically enriched in HSP 68-70.

Chromatin was altered within 10 minutes after shift of Reuber H35 cells to 43°C (temperature increased from 37°C to 43°C in 5 minutes). This early alteration was reflected by alterations in endogenous phosphorylation of isolated NHCP's. A specific enhancement was found for proteins with molecular weights of 70.000 and 95.000 dalton. When the heat treatment was extended, endogenous phosphorylation of isolated NHCP's was further changed. ^{32}P incorporation further increased for protein of Mr 95.000, while it decreased for protein Mr 70.000. The initiation of these early alterations in chromatin preceded (a) the increase of HSP synthesis, and (b) attachment of newly synthesized HSP 68-70 in the chromatin fraction.

We do not know yet the underlying mechanisms for alteration of endogenous phosphorylation capacity of isolated NHCP's. The change in extent of NHCP phosphorylation may be interpreted in several ways (26) (a) Change in the molecular species of the endogenous substrates, (b) Changes in the number of sites of phosphorylation available in vitro and (c) Increase in a protein kinase specific for endogenous substrates.

The early alterations in chromatin, as reflected by alterations of endogenous NHCP phosphorylation, probably precede the HSP gene transcription. In preliminary experiments we determined the period of HSP-mRNA synthesis during and after heat treatment (also 30 minutes 43°C) by addition of actinomycin D. When actinomycin D was added within 20 minutes from the initiation of heat treatment, enhancement of HSP synthesis was totally prevented, indicating that no HSP gene transcription takes place

during the first 20 minutes of treatment.

After heat treatment HSP's appear in the cytoplasm. HSP 68-70, but none of the other HSP's, were also found associated with chromatin. These proteins are specifically released from the chromatin at later times. This suggests that binding properties between chromatin and HSP 68-70 changes during and after heat shock. Evidence for altered binding between chromatin and specific proteins has been found in a few other studies (27,28). Moreover, SV 40 T antigen and c-myc protein, loosely associated with the nucleus before heat treatment, were found immobilized afterwards (29).

In a number of studies, a correlation between heat induced synthesis of HSP's and development of thermotolerance has been described (30-33). Whether the accumulation of HSP 68-70 in the chromatin fraction is a prerequisite for development of thermotolerance is not clear. Our data demonstrate that for the maintenance of the thermotolerant state the continuous presence of HSP 68-70 in the chromatin fraction is not needed. But thermotolerance might be explained by an immediate association of existing HSP's with chromatin upon heat shock. This would be consistent with experimental data which deal with distribution of HSP's upon fractionated heat treatment of Drosophila cells. In these cells HSP 70 can shuttle between nucleus and cytoplasm (13,15,34). During heat shock the protein was concentrated in the nucleus. During the recovery period HSP 70 left the nucleus again and was distributed throughout the cytoplasm. However when a second heat shock was applied it was rapidly transported back to the nucleus. It is more likely therefore to suggest that thermotolerance depends on (a) the status of chromatin as reflected in NHCP's and (b) the level of cellular HSP 68-70.

Further knowledge of the sequence of heat induced alterations in chromatin, association of existing - and newly synthesized HSP 68-70, is needed for sorting out the biochemical and molecular events relevant for expression of thermotolerance.

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Chapter 9

Summary and discussion

This thesis deals primarily with the effects of heat shock on the proliferative capacity of synchronized cells and with the molecular mechanisms involved in getting over a disturbance of cell proliferation caused by temperature shock. Attention has been focused on the following main questions in heat stress physiology: (a) What are the physiological parameters influencing cellular heat sensitivity, (b) What are the critical targets for heat action and (c) What are the molecular mechanisms involved to encounter a heat shock. In order to relate heat induced effects on proliferation with general or specific biochemical and molecular events relevant for cell division these questions were posed for synchronized cells.

The effects of heat shock on the proliferative capacity of cells were shown to depend on (a) the thermal history of a cell, (b) the position of the cell in the cell cycle and (c) environmental factors (serum factors). The effects of heat on cell proliferation were modified by hypo- or hyperthermic preincubation. As was demonstrated in studies on cell survival, immediately after shift to hypothermic temperatures neuro-2A cells became more sensitive to hyperthermia (Chapter 3). The rate of thermosensitization depended on (a) hypothermic temperature, (b) length of hypothermic preincubation and (c) the cell cycle phase at the moment of shift to hypothermic temperature. The mechanism of thermosensitization by hypothermic preincubation is still unknown. Preliminary data suggest that neither the

constitutive HSP synthesis nor the process leading to the induction of HSP synthesis upon heat shock, become affected by hypothermic preincubation. Probably, alteration of heat sensitivity by hypothermia is not directly related to alteration of HSP synthesis. In Chapter 3 it was further demonstrated that the effect of heat treatment on cell survival is related to the cell cycle. Cells in late S and early G2 phase were more sensitive to heat shock than were G1/early S phase cells.

Alteration of heat sensitivity throughout the cell cycle was further described in Chapter 4. Mitotic delay by a nonlethal heat treatment was larger in late S/G2 phase cells than in G1 phase cells. Cell cycle progression of heated cells became thermotolerant to a second incubation at increased temperature but thermotolerance developed more rapidly in G1 than in late S/G2 phase cells. These data may be compatible with results of Read et al. (1) who studied the heat induced development of thermotolerance for cell survival in cells at different parts of the cell cycle. They demonstrated that the increase of thermoresistance after an initial heat treatment was more rapid in G1- than in G2 phase cells.

In Chapter 6 the question was raised whether differences in HSP synthesis can be held responsible for differences in heat sensitivity throughout the cell cycle as were observed for induction of mitotic delay and cell killing. It has been concluded that the protection against heat damage by HSP's is more pronounced in G1 phase cells than in late S/G2 phase cells. Nevertheless, basal and heat induced HSP synthesis did not alter throughout the cell cycle.

Several explanations are possible for these data. One explanation might be that HSP's synthesized by G1 phase cells are different from those synthesized by late S/G2 phase cells, whereas the critical targets for heat action are the same in these cell cycle phases. According to this hypothesis one has to assume that HSP's synthesized in G1 phase cells alter when these cells progress through the cell cycle, because in Chapter 4 it was demonstrated that expression of tolerance for heat induced mitotic delay diminished when heated G1 phase cells progressed through S into G2 phase. Decrease of thermoresistance occurred while HSP levels remained high (Chapter 6). A single target might be responsible for occurrence of mitotic delay after heat shock (and/or cell killing), while HSP's protect this target to different extents throughout the cell cycle. In fact, HSP's can be modified by phosphorylation (2), methylation (3), glycosylation (4) and/or ADP-ribosylation (5). However the functional significance of these

modifications is totally unknown. Neither is known whether modification of these proteins is related to cell cycle. A second obvious interpretation of these results is that HSP's synthesized in G1 phase cells are similar to those synthesized by late S/G2 phase cells whereas the critical targets for heat action are different in these cell cycle phases. In that case at least two targets which are differentially exposed throughout the cell cycle, are responsible for the occurrence of mitotic delay after heat shock (and/or cell killing) and HSP's protect these targets unequally.

Another parameter which has been shown to affect heat sensitivity is the presence of serum during heat treatment (Chapter 5). Serum protects neuro-2A cells against heat induced alterations of cell cycle progression as well as against heat induced cell death. However, protection by serum was observed in G1- but not in late S/G2 phase cells. Serum affected heat induced alterations of proliferation immediately i.e. removal of serum caused an immediate sensitization of cells. It is unlikely that HSP synthesis is involved in this rapid modification of heat sensitivity but definitive proof is lacking. Protective effects of serum against heat damage were also observed in other cell systems. Serum but also specific growth factors protected Swiss mouse 3T3 cells against heat induced morphological alterations (6). Immediately after serum addition heat resistance developed. Cycloheximide did not affect the increase of heat resistance indicating that a non-protein compound is involved in serum protection against heat.

In Chapter 7 we described the rapid increase of heat resistance after serum stimulation of resting Reuber H35 cells. Immediately upon serum addition the cells became resistant to heat induced rounding-up and to heat induced cell death. This early increase of heat resistance is probably not related to increase of HSP synthesis as was demonstrated by assaying the relative synthesis rates of individual HSP's.

There is some evidence that chromatin is the target for heat killing (7). In the last two chapters (Chapters 7 and 8) thermoregulation at chromatin level was considered. Chapter 7 deals with growth stimulation of resting Reuber H35 cells by serum containing medium. Thermoresistance increased upon growth stimulation and in parallel structural and molecular properties of chromatin became resistant. We speculated about the molecular basis for increased thermoresistance after growth stimulation. Alteration of the thermoresistance of chromatin coincides with specific alterations within the chromatin fraction as was demonstrated by in vitro phosphorylation of isolated NHCP's. These alterations seem to precede the

onset of HSP synthesis. A heat treatment caused similar changes in the NHCP fraction (Chapter 8). These heat induced events in chromatin preceded (a) enhancement of HSP synthesis, (b) attachment of newly synthesized HSP 68-70 in the chromatin fraction and (c) development of thermotolerance. Preliminary data indicate that also preexisting HSP 68-70 becomes associated with the chromatin. Attachment of preexisting HSP 68-70 started to occur during heat treatment while newly synthesized HSP 68-70 was found associated to the chromatin afterwards.

It is likely to suggest that thermotolerance depends on (a) the status of chromatin as reflected in endogenous phosphorylation of isolated NHCP's which might be important for binding of HSP's and (b) the level of HSP's.

These data may give a molecular clue for explaining:

a) alteration of thermosensitivity without alteration of HSP synthesis as observed in Chapter 3 for hypothermic treatment, in Chapters 5 and 7 upon alteration of serum concentration and in a number of other studies (8-12). Under these conditions alterations in the physiological status of the cell may be accompanied with alterations in chromatin while HSP synthesis does not change. As a result of changes within chromatin, preexisting HSP 68-70 might shuttle between cytoplasm and nucleus and be part of the mechanism involved in altering heat sensitivity.

b) Alteration of thermosensitivity which is linked to alteration of HSP synthesis (and NHCP's) as described in Chapter 8 and in some other studies (13-16).

c) Alteration of heat sensitivity throughout the cell cycle. We concluded in Chapter 6 that the role of HSP's in protection against heat damage is more pronounced in G1 phase cells than in late S/G2 phase cells while basal and heat induced HSP synthesis did not alter throughout the cell cycle. An explanation for these observations might be the alteration of the chromatin status throughout the cell cycle leading to altered interaction with HSP's.

Evidence for a role of HSP 68 in heat protection at chromatin level came from recent studies of Pelham. He localized HSP 68 in the nucleus but after heat shock it was particularly concentrated in nucleoli (17). The structure of nucleoli alters upon heat shock. Pelham demonstrated that the recovery of normal morphology occurs much more rapidly in cells which have been preloaded with HSP 68 by transfecting them with a plasmid which expresses the gene constitutively. The recovery does not require

protein synthesis and the repair of the nucleoli thus appears to be mediated by HSP 68. He further demonstrated by transfection of mutant genes that there is a relation between the ability of HSP 68 to associate with the chromatin fraction and the ability to speed the recovery of nucleoli (18). These properties of HSP 68 were disturbed when 2-deoxy-glucose was present during heat shock, which indicates that alteration of the metabolic status of a cell leads to altered functioning of the protein. These data may be compatible with the hypothesis that thermoresistance depends on the level of HSP's but also on the chromatin status as reflected in NHCP's.

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Samenvatting

Dit proefschrift beschrijft enerzijds de effecten van hyperthermie op de proliferatie capaciteit van gesynchroniseerde zoogdiercellen en anderzijds de moleculaire mechanismen die betrokken zijn bij de bescherming van een cel tegen hyperthermie. Om de effecten van hyperthermie op de celproliferatie te kunnen relateren aan algemene of specifieke biochemische en moleculaire processen die nodig zijn voor celdeling werden deze studies verricht aan gesynchroniseerde cellen. Er wordt aandacht besteed aan drie belangrijke vragen in de heat stress fysiologie: (a) wat zijn de fysiologische parameters die de warmtegevoeligheid van een cel bepalen, (b) wat zijn de kritische processen c.q. structuren die verstoord worden door hyperthermie, en (c) wat zijn de moleculaire mechanismen die de cel beschermen tegen een heat shock.

Hoofdstuk 2 geeft een overzicht van de meest recente literatuur met betrekking tot dit onderzoeksgebied. Een interessant aspect van de heat stress fysiologie is de waarneming dat een cel na een eerste warmtebehandeling resistent kan worden voor een tweede warmtebehandeling. Deze toename in warmteresistentie wordt thermotolerantie genoemd. Een aantal waarnemingen duiden op de betrokkenheid van bepaalde eiwitten, de heat shock proteins (HSP's), bij het optreden van thermotolerantie en bij de regulatie van de warmtegevoeligheid van een cel in het algemeen. De synthese van HSP's is verhoogd na een heat shock. Ondanks dat er uitgebreid onderzoek is gedaan naar de synthese en cellulaire distributie van HSP's zijn er tot nu toe geen specifieke functies voor deze eiwitten bekend.

In de hoofdstukken 3, 4 en 5 wordt getoond dat het effect van een heat shock op de proliferatie capaciteit van cellen afhangt van (a) de voorbehandelingstemperatuur, (b) de positie van een cel in de celcyclus en (c) omgevingsfactoren zoals serum. Het effect van een heat shock wordt gemodificeerd door hypo- of hypertherme voorbehandeling. In overlevingsproeven werd aangetoond dat de warmtegevoeligheid van Neuro-2A cellen toeneemt, direct nadat deze bij hypotherme temperaturen worden geplaatst (Hoofdstuk 3). De snelheid van thermosensitivering hangt af van (a) de hypotherme temperatuur, (b) de tijdsduur van hypotherme preincubatie en (c) de celcyclus fase op het moment dat de cellen met hypothermie behandeld worden. Bovendien blijkt het effect van een heat shock op de celoverleving gerelateerd te zijn aan de celcyclus. Cellen in de late S fase of vroege G2 fase zijn gevoeliger voor een warmtebehandeling dan G1- of

vroege S fase cellen.

Verandering van warmtegevoeligheid tijdens de celcyclus wordt nader beschreven in hoofdstuk 4. Het mitose-uitstel ten gevolge van een niet-lethale heat shock is groter in late S/G2 fase cellen dan in G1 fase cellen. Na een eerste heat shock wordt de celcyclus progressie van Neuro-2A cellen tolerant voor een tweede shock. Deze tolerantie ontwikkelt zich sneller in G1 dan in late S/G2 fase cellen.

Een andere parameter die invloed heeft op de warmtegevoeligheid van een cel is de aanwezigheid van serum-bevattend medium tijdens de warmtebehandeling. Serum beschermt Neuro-2A cellen tegen hitte-geïnduceerde veranderingen in celcyclus progressie alsook tegen hitte-geinduceerde celdood. Dit beschermend effect werd wel in G1 fase cellen waargenomen, maar niet in late S/G2 fase cellen. Serum beinvloedt de hitte-geïnduceerde veranderingen in celproliferatie direct: verwijdering van serum veroorzaakt een directe sensitivering.

In de hoofdstukken 6, 7 en 8 wordt de vraag gesteld wat de moleculaire basis is voor de regulatie van warmtegevoeligheid zoals waargenomen in de voorafgaande drie hoofdstukken. De resultaten zoals gepresenteerd in hoofdstuk 6 duiden op een functie van HSP's bij de proliferatie van Neuro-2A cellen na een heat shock. Er werden aanwijzingen gevonden dat HSP's de G1 fase cellen beter tegen heat shock beschermen dan de late S/G2 fase cellen. De basale HSP synthese en de door heat shock geïnduceerde HSP synthese veranderen niet tijdens de celcyclus.

Er zijn aanwijzingen dat schade in het chromatine verantwoordelijk is voor hitte geïnduceerde celdood. In de hoofdstukken 7 en 8 wordt de regulatie van de warmtegevoeligheid op chromatine nivo bestudeerd. Hoofdstuk 7 handelt over groeistimulatie van rustende Reuber H35 hepatoma cellen door serum-bevattend medium. Na groeistimulatie neemt de thermoresistentie van cellen toe en parallel hieraan worden ook structurele en moleculaire eigenschappen van het chromatine thermotolerant. We speculeren over de moleculaire basis voor de toename van thermoresistentie na groeistimulatie. De verandering in thermoresistentie van chromatine valt samen met specifieke veranderingen in de chromatine fractie zoals aangetoond wordt d.m.v. 'in vitro' fosforilering van geïsoleerde Non-Histone Chromosomal Proteins (NHCP's). Deze veranderingen gaan aan de verhoging van HSP synthese vooraf. Een heat shock veroorzaakt vergelijkbare veranderingen in de NHCP fractie (Hoofdstuk 8). Deze hitte geïnduceerde veranderingen in het chromatine worden gevolgd door (a) een verhoging van de HSP syn-

these, (b) binding van nieuw gesynthetiseerd HSP 68-70 aan het chromatine en (c) de ontwikkeling van thermotolerantie. Recente resultaten laten zien dat ook constitutief gesynthetiseerd HSP 68-70 geassocieerd raakt met het chromatine. Binding van constitutief gesynthetiseerd HSP 68-70 vindt tijdens de heat shock plaats terwijl nieuw gesynthetiseerd HSP 68-70 erna bindt. Mogelijk dat thermotolerantie afhangt van (a) de toestand van het chromatine, zoals gereflecteerd in de endogene fosforilering van geïsoleerde NHCP's, welke belangrijk kan zijn voor binding van HSP's en (b) de hoeveelheid HSP's die in de cel aanwezig is. In de slotdiscussie wordt aan de hand van dit idee een moleculaire verklaring gegeven voor:

a) Verandering van warmtegevoeligheid zonder verandering in HSP synthese zoals waargenomen in hoofdstuk 3 voor hypothermie behandeling, in de hoofdstukken 5 en 7 na verandering van serum concentratie en in een aantal recente artikelen.
b) Verandering van warmtegevoeligheid welke gekoppeld is aan verandering van HSP synthese (en NHCP's) zoals beschreven in hoofdstuk 8 en in een aantal artikelen.
c) Verandering van warmtegevoeligheid tijdens de celcyclus.

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Curriculum vitae

De schrijver van dit proefschrift werd 31 december 1956 geboren te Rijen. Het voorbereidend wetenschappelijk onderwijs volgde hij aan het St-Odulphus lyceum te Tilburg, alwaar in 1975 het diploma Atheneum B werd behaald.

In 1975 startte hij met de studie biologie aan de Rijksuniversiteit Utrecht. Het kandidaatsexamen B4 (Biologie met als tweede hoofdvak scheikunde) werd in september 1979 afgelegd, het doctoraalexamen werd in december 1981 behaald. De hoofdvakken waren Immunologie (Prof.Dr. R. Ballieux, Dr. C. Heijnen) en Moleculaire Celbiologie (Prof.Dr. H.O. Voorma, Dr. R. van Wijk) en de nevenrichting was Tumorpathologie (Prof. Dr. W. den Otter, Dr. H. Dullens, Dr. K.P. Dingemans).

Op 1 januari 1982 maakte hij een aanvang met een promotieonderzoek bij de vakgroep Moleculaire Celbiologie, projectgroep Regulatie van de Genexpressie onder (bege)leiding van Prof.Dr. H.O. Voorma en Dr. R. van Wijk. Dit onderzoek werd mogelijk gemaakt door een 4-jarig promotie-assistentschap van de Rijksuniversiteit Utrecht.

STELLINGEN

I

Uit de beschrijving, die LaThanque geeft van een heat shock eiwit aangeduid p68, is het niet op te maken of het hier een nieuw heat shock eiwit betreft of één van de reeds eerder beschreven eiwitten HSP 68 of HSP 70.

LaThanque, N.B. (1984) EMBO J. 3:1871-1879.

II

In de experimenten van Li en Laszlo worden geen nieuwe argumenten aangedragen die pleiten voor een functionele rol van HSP's bij de bescherming van cellen tegen hyperthermie.

Li, G.C. and Laszlo, A. (1985)
J. Cell. Physiol. 122:91-97.

III

Sciandra en Subjeck hebben voor het aantonen van het fenomeen 'protectie van translatie', bij gefractioneerde hitte-behandeling, onvoldoende controles gebruikt.

Sciandra, J.J. and Subjeck, J.R. (1984)
Cancer Res. 44:5188-5194.

IV

Darveau et al. demonstreren in hun titel, experimentele opzet en evaluatie van de resultaten een a priori aanname van de te bewijzen stelling, namelijk dat het de 5'- niet vertaalde sequentie is die c-myc m-RNA translatie beïnvloedt.

Darveau, A., Pelletier, J. and Sonenberg, N.
(1985) Proc. Natl. Acad. Sci. U.S.A. 82:2315-2319

V

De methode voor detectie van oppervlakte-gebonden liganden d.m.v. vriesbreek autoradiografie zoals beschreven door Carpentier et al. is geen verbetering ten opzichte van reeds bestaande technieken.

Carpentier, J.L., Brown, D., Iacopetta, B. and
Orci, L. (1985) J. Cell Biol. 101:887-890.

VI

Klonering van het full-length cDNA van de chimere bcr/c-abl messenger zou kunnen leiden tot de eerste directe aanwijzing dat de vorming van het bcr/c-abl gen d.m.v. translocatie en het optreden van chronische myeloide leukemie (CML), oorzakelijk verbonden zijn.

VII

Het opleggen van kortingen op uitkeringen bij onvoldoende solliciteren levert geen structurele bijdrage aan het verminderen van de werkloosheid.

VIII

De stelling dat de kans op een technisch mankement in een space-shuttle of kruisraket klein is, hoeft op zich niet onjuist te zijn. De stelling dat space-shuttles en kruisraketten volledig controleerbaar zijn, is echter volstrekt onjuist.

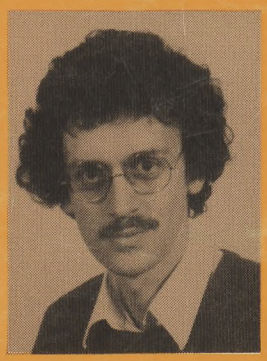
IX

Het wettelijk toestemming geven aan fietsers om bij het rechtsafslaan op een kruispunt door rood stoplicht te rijden, kan een belangrijke bijdrage leveren aan het terugdringen van het aantal verkeersovertredingen.

X

Er zijn geen aanwijzingen dat de toenemende stress op het einde van een promotieperiode gepaard gaat met een verhoogde tolerantie van de promovendus.

Proefschrift van Guus van Dongen
Effects of Hyperthermia on Mammalian Cells
Utrecht, 12 mei 1986.



Effecten van hyperthermie op zoogdier cellen

Hyperthermie, het verhogen van de temperatuur van een deel van het lichaam, wordt de laatste jaren steeds vaker als kankertherapie toegepast, meestal in combinatie met bestraling. Het is voor de klinische toepassing van hyperthermie noodzakelijk dat bij behandeling de tumor verdwijnt terwijl het normaal weefsel niet beschadigd wordt. In dat verband is het belangrijk om te weten welke factoren de warmtegevoeligheid van cellen beïnvloeden.

Een practisch probleem bij de klinische toepassing van hyperthermie vormt het vermogen van tumorcellen om, bij herhaalde hyperthermie behandeling, warmteongevoelig te worden: het verschijnsel van thermotolerantie. De moleculaire basis voor thermotolerantie is vrijwel onbekend ofschoon een aantal waarnemingen wijzen op de betrokkenheid van bepaalde eiwitten, de zogenaamde heat shock eiwitten of HSP's, waarvan de synthese na heat shock verhoogd is.

In dit proefschrift wordt aandacht besteed aan drie belangrijke vragen vanuit de heat stress fysiologie: (a) wat zijn de fysiologische parameters die de warmtegevoeligheid van een cel bepalen, (b) welke kritische processen c.q. structuren in een cel worden door hyperthermie verstoord en (c) welke moleculaire mechanismen zijn betrokken bij de bescherming van een cel tegen een heat shock.