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Heat shock and the role of the HSPs during neural plate induction in early mammalian CNS and brain development

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Abstract. We have investigated the early development expressional of the heat shock protein genes (*hsps*) and HSP synthesis and their role during neuroectoderm induction, differentiation and early CNS formation. The expression and kinetics of 90, 73/71, 47 and 27 HSPs on neuroectoderm differentiation was compared under normal and stressed conditions. The role of HSPs on neuroectoderm cell fate including thermotolerance and apoptosis using a whole in vitro embryo culture system was studied. Hsp expression appears closely linked in early mammalian development to critical differentiation and proliferation stages in early brain and heart formation. The hsps are developmentally activated around blastula stage and HSPs are constitutively expressed at high levels during neural tube closure and are heat shock responsive. Using both Northern analysis, confocal microscopy and whole mount in situ hybridisation we have identified the mRNA hsp transcripts and HSPs during organogenesis. HSPs were detected during neuroectoderm cell induction and differentiation with the hsp mRNA being tightly regulated during the cell cycle of neuroectoderm especially at early fore-, mid-, hindbrain and heart formation. The 'chaperone' functions of the HSPs are well known, recently during gastrulation the HSP47 and 27 have been shown to specifically bind and fold to nascent collagen and actin molecules respectively. This role is essential for the formation of the basement membrane, extra cellular matrix and neural crest migration during neural plate development. HSP function was observed by using anti-sense strategy, short '5 anti-sense cDNA' hsp oligonucleotides inhibited hsp expression during gastrulation in the whole embryo cultures. The developmental activation of the heat shock element (HSE) is essential to our understanding of the HSPs role in neuronal cell fate. Using specific polyclonal antibodies to HSF1 and 2 (Dr Nakai, Kyoto University) the expression of heat shock factors (HSFs) during neuroectoderm differentiation was examined. Using Western analysis, confocal microscopy and flow cytometry HSF1 and 2 were identified and studied under both normal and heat shocked conditions. During gastrulation higher levels of HSF1 and 2 were identified in the neuroectoderm layer especially in regions of the fore-, mid- and hindbrain. The heat shock response and activation of the HSPs 90, 70, 47 and 27 families have been correlated with HSF1 and 2. The HSF1 appears to be present in all early embryonic cells but appears not to bind to the HSE untill early head fold stage at gastrulation when the presence of HSF2 is observed. During neuroectoderm differentiation the activation of HSF1 and 2 appears to correlate with high constitutive expression of many of the *hsp*s specificially *hsp*90, 73, 71, 47 and 27 being tightly regulated by the cell cycle at neurulation.
Key words. Heat shock proteins (HSP); embryo culture; developmental defects; thermotolerance; cell cycle regulation.

Introduction

Hyperthermia and human studies

Records from many sources indicate that approximately 3% of newborn children have a developmental defect requiring medical attention, and approximately one-third of these conditions can be regarded as life threatening. With increasing age, the number of defects detected is more than doubled [1]. The emotional and financial cost of birth defects is enormous; approximately one-half the children in hospitals are there because of a birth defect. In most instances the cause of the defect remains unknown, about 25% is genetic in origin and less than 10% can be ascribed to a teratogenic agent.

Although Shepard [1] lists only 36 known and 13 possible human teratogens, there are at least 1200 agents known to cause defects in animals. Human teratogens have usually been detected by clinical observation or less frequently by epidemiological surveys of children with defects and subsequently, the mechanisms and conditions of teratogenicity have been studied in animals. Hyperthermia is an exception to this generalisation. Animal research showed heat shock was teratogenic in

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many species, including primates, and the conditions and mechanisms of the action were defined in animals before a systematic study of its effects was made in humans. Recently subsequent human clinical observations and epidemiological studies indicated that hyperthermia is also a human teratogen although general acceptance that a naturally occurring environmental agent such as heat could be teratogenic has been slow. The volume of clinical, epidemiological and experimental evidence on heat as a teratogen is now very large (review by Edwards et al., see ref. 2)

Hyperthermia and the normal body temperature

Hyperthermia refers to a higher than normal body temperature with the induction of a characteristic cellular heat shock response and the induction of the heat shock proteins (HSP) interrupting and altering the physiological homeostatic mechanisms of the cell. It can be caused by many conditions and agents including febrile infections, heavy exercise, a hot and humid environment, exposure to saunas, hot tubs, electromagnetic radiations, microwaves and ultrasound and many drugs (such as phenothiazines, amphetamines, tricyclic antidepressants, antihypertensive drugs, cocaine, LSD), and organic compounds (such as organophosphates, dinitrophenols) particularly when taken in a hot environment [3]. Most deep core body temperatures of mammals fall between 37 °C and 40 °C and remain relatively stable under widely variable environmental conditions of cold and heat. Normally, the deep (core) body temperature is lowest during periods of inactivity and sleep, increasing on arousal, physical activity and feeding, the usual range being 1 °C on either side of the average for the species.

Teratogenesis

The *hsp* genes and HSPs have been highly conserved through evolution in all species studied from humans through to bacteria. The relatively high and stable body temperatures of mammals confer many advantages in survival compared with poikilothermic animals. It could be inferred that evolutionary pressures should result in even higher body temperatures unless prevented by some biochemical or physiological barriers. It has been suggested that the deleterious effects of heat on spermatogenesis [4] and cellular proliferation in embryos [5] are two such barriers. Although it has been recognised for many years, that heat shock during early pregnancy causes increased rates of embryonic resorption in many species of animals, the other effects of the heat shock response in causing defective development have only recently been explored in detail.

The type of defects caused by heat shock depends largely on the stage of embryonic development at the time of exposure and the severity depends largely on the 'dose' of heat, which is a product of the temperature elevation and the duration of elevation. Exposure of pre-implantation embryos is commonly followed by embryonic death and resorption in a wide range of species [6] and these deaths can be caused by quite modest temperature elevations. More severe exposures of post-implantation embryos or fetuses are required to cause embryonic or fetal death and resorption or abortion. After implantation, severe exposures are followed by prenatal death and resorption or abortion while defective embryonic development follows less severe exposure. Experimental production of defects by heat shock, with low levels of resorptions and abortion, can be achieved by maintaining rigid control of the environmental temperature and the duration of exposure with frequent monitoring of the maternal temperature. During non-experimental exposures such as in infectious fevers, saunas, hot tubs and drugs, there is not the same level of control of the dose of heat and it has been suggested that abortion could be the most common adverse outcome in women [7].

The 'dose' of heat is a product of the elevation of temperature and the duration of elevation. There is no simple measure for dose in humans, but a number of authors have approached the problem of threshold dose by finding the shortest exposure time at a given elevation of temperature for the production of a defect [8–10]. These studies show that as the exposure temperature is increased, the time required to cause a defect is reduced logarithmically.

Human studies have shown a number of defects to be associated with a maternal hyperthermic episode. Neural tube defects were prominent [11–13] and it was estimated that approximately 10% were due to maternal hyperthermia at the time of tube closure [7]. Other defects were microphthalmia [14, 15], microcephaly, neuronal heterotopias, micropenis, micrognathia, midface hypoplasia, cleft lip/palate, external ear anomalies, mental deficiency, hypotonicity, neurogenic contractures including talipes and arthrogryposis, seizures [16], Moebius syndrome [17] and Hirschsprung disease [18].

Experimental heat shock studies

Defects have been produced experimentally by hyperthermia caused by a variety of methods, in chickens by elevated temperatures of incubation [19–21], pregnant rats in hot air incubators [22–24], exteriorised pregnant rat uterine horn immersed in hot water [25]; diathermy [26]; abdomen of pregnant rat immersed in warm water [9, 27]; heated embryo cultures [5, 28, 29]; radiofrequency radiation [8, 30, 31]; ultrasound in cultured embryos [32], pregnant mice in hot air incubators [33, 34]; abdomen of pregnant mice immersed in warm water [35–37] and exposure to microwaves [38]; pregnant guinea pigs in hot air incubators [23, 39, 40], hamsters

in hot air incubators [41–43], rabbits [44], fever induced by injection of milk endotoxin induced fever in sheep [45] and in hot air chambers [46], pigs in hot air chambers [47], and monkeys [48, 49] in hot air chambers. In a number of studies there was evidence of synergism between minimally teratogenic doses of heat and minimally teratogenic doses of other agents including vitamin A [43], arsenic [42], lead [50], ultrasound [32], alcohol [37] and endotoxins [51].

The defects induced experimentally by heat shock have a wide developmental spectrum corresponding to the interruption of cell differentiation during organogenesis at the relevant stages of development. These include neural tube defects, cranio-facial defects, microphthalmia, heart defects, coloboma, kyphosis, scoliosis and skeletal defects when exposed about the time of neural tube closure. Cataract, talipes, hypodactyly, micrencephaly, renal and dental agenesis, exomphalos, cranial nerve defects, and behavioural abnormalities, follow exposure at later stages of embryogenesis [52].

The experimental in vivo studies provided information about the types of induced defects and their approximate stages of greatest sensitivity during embryogenesis, mechanisms of causing defects and approximate thresholds of maternal temperature elevation and duration of elevation. The periods of susceptibility to defects can be quite brief and vary in the wide variability of embryonic development at a given conceptual age, even within litters, and they appear to result in irregular expression of the effects of heat shock especially following a single brief exposure [2]. In contrast, by using embryo culture techniques, the stage variability can be minimised by selecting embryos at a precise, identifiable stage of development, for example, the early pre-somite head fold stage at 9.5 days [10], embryos at 6–10 somites with yolk sac circulation and heart beat [53] or at 10–12 somites [54].

Another problem with in vivo studies is the difficulty in estimating the heat shock delivered through the mother to the em bryo and estimating via rectal temperature of the mother during the heating and cooling phases. In embryo culture studies, it is possible to apply a precise temperature to the embryo, with short accurate heat shock maintained for a precise duration. In clinical studies, it is often difficult to separate the direct and indirect effects of heat shock on the embryo. In instances such as viral infection causing maternal fever, a teratogenic effect could be due to viral infection of the embryo, metabolic changes in the mother affecting the embryo or direct action of hyperthermia on the embryo. The embryo culture technique can eliminate the maternal effects.

In vitro studies

Cockroft and New [28, 29] were the first to use elevated temperatures on rat embryos in culture. Embryos of the CFGB strain were explanted on day 9.5 of gestation (egg cylinder, early neural fold stage) and cultured for 2 days at 38 °C (controls), 40 or 41 °C. Other heated groups were exposed for 12 or 23 h of the culture period to 40, 40.5 or 41 °C, and for the rest of the culture at 38 °C. Culture at 40 °C for 2 days resulted in overall growth similar to controls but blood circulation was disturbed in a proportion and about half had small developmental abnormalities, including a number with apparent microcephaly. Two methods were used to clarify the effect of culture at 40 °C on brain and head growth. In the first method, embryos grown at 38 °C were paired with an embryo at 40 °C on the basis of total body protein content. The amount of protein in the heads was measured separately. The mean total body protein content of each group was identical but the head:body ratio of the 40 °C group was significantly less than that of the control group. The other method matched individual embryos from each group on the basis of crown-rump length and measured the length of the head and the length, height and width of the telencephalon. The length of the head and the length and height of the telencephalon were significantly less in the 40 °C group compared with the 38 °C group.

At 40.5 °C over the 2 days, growth was retarded, somite numbers were less and over half the embryos were obviously microcephalic. Exposure to 41 °C for the whole culture period caused severe problems including very poor growth and somite formation, microcephaly, neural tube defects, enlarged hearts and pericardial oedema. In experiments in which embryos were cultured at 41 °C for 12 h periods between 9.5 and 11.5 days, development was most vulnerable to damage during late day 9 to early day 10.

Comparisons of results of in vitro with in vivo exposures from the same laboratory are few, but valuable. By exposing day 10 (10–12 somite) rat embryos in vitro, after 2 h at 38 °C, to temperatures of 42 or 43 °C for 10–25 min, Kimmel et al. [54] found a dose-related general inhibitory effect on growth in all systems, especially systems that were developing most rapidly at the time of the exposure. The embryos were evaluated on day 11 using a modified Brown and Fabro [55] method. A similar exposure in vivo on day 10 [54] resulted at term, in a very high rate of similar skeletal defects of the thoracic vertebrae and ribs, but fewer defects of the head. The authors suggested that this disparity in response could be due to a greater dose of heat in the in vitro system in which embryos were kept at 42 °C for at least 10 min which was twice as long as the in vivo exposure. Their results showed that in the in vivo experiments, it took at least 50 min to achieve the required temperature. When estimating the dose of heat in vivo, the heating-up and cooling-down phases should be considered for inclusion in the dose because a significant dose of heat can be delivered during these periods.

The durations of the heating and cooling phases in vitro usually occupy only 2–3 min each. It also appears possible that the disparate results could be due to a high prenatal mortality of severely defective embryos in vivo. In addition, the authors pointed out that in vitro embryos might have recovered from many of the minor developmental deficits by subsequent compensatory growth in utero.

Angles et al. [32] used 9.5 day rat embryos in culture to test the effects of 5, 15 or 30 min of pulsed ultrasound (SPTA intensity of 1.2 W/cm^2, similar to diagnostic Doppler). Examination at 11.5 days showed that insonation for 30 min caused only a small deficit in somite number, equivalent to a 2 h delay in development. Embryos given similar insonation for 15 min in culture medium at 40 °C (+1.5 °C), had a significant reduction in total protein and also in the head:body surface area ratio. Insonation for 15 min at 38 °C or culture at 40 °C for 15 min alone had no detectable effect on development.

Nakashima et al. [56] used cultured 8 day (3–5 somite) mouse embryos (C57BL/6 Cr Slc female X C3H/He Slc male strains) to compare the individual and combined effects of X-irradiation and hyperthermia on early development. Dose response studies showed that 0.3 Gy of X rays given at 1 h after the start of culture caused no detectable changes in development at the end of the 40 h culture period. Doses between 0.6–2.0 Gy caused progressively more damage, with 10% of embryos given 0.6 Gy showing microphthalmia and 77% of embryos given 2.0 Gy showing open neural tubes and 100% microphthalmia. Exposure to 43 °C for 5 min caused no defects and there was significant but minor retardation of protein accumulation and somite formation. Embryos exposed for 10 min had 28% open neural tube and 78% microphthalmia, increasing to 100% open tube, 100% anophthalmia and 100% heart defects when given 20 min. Control embryos were incubated at 38 °C. In terms of defective development, the 10 min exposure to 43 °C corresponded roughly to 1.2 Gy of X rays. A combination of 0.3 Gy and 5 min at 43 °C, which were not teratogenic when given individually, caused significant microcephaly, 17% microphthalmia and 11% heart defects.

The precision of the embryo culture experiments clarified a number of points which had not been possible using pregnant animals. They showed that hyperthermia acted directly on the embryo and that defects were not mediated through toxic maternal changes [57], although the maternal reaction might modify the embryonic response. The studies also confirmed the extreme sensitivity to retardation of brain growth. It is uncertain whether the deficit in head size and forebrain of the 11.5 day rat embryos would be permanent. However, the deficit in brain size found in 30 day guinea pig embryos after heating on day 21 persisted to adult life [58, 59]. Also, retardation of growth of the head resulted from a 2 °C elevation of temperature, which is well within the range occurring in fevers and other environmentally induced elevations. Embryos cultured at 40.5 °C (+2.5 °C) showed more severe microcephaly and retardation of development.

Because of the different experimental conditions employed in various rat embryo culture and in vivo studies, it is difficult to draw conclusions on a number of factors. One factor has been the definition of the threshold dose causing defective embryonic development. Is the threshold best described by the actual temperature achieved and duration at that temperature, or the elevation of temperature above normal and duration at that elevation? The threshold has been estimated in vivo in 21 day guinea pig embryos as a spike to 41.5–42 °C (2–2.5 °C above the normal temperature, 39.5 °C), rising and falling over a period of about 80 min. Cockroft and New [28] showed that incubation at 40 °C (2 °C above the control temperature) caused retardation of head growth of rat embryos, but the exposure was for 2 days. This result indicated that the threshold of temperature related better to the elevation above normal (2–2.5 °C) rather than to the actual temperature achieved (40 °C in rats and 41.5 °C in guinea pigs). Germain et al. [9] found other thresholds for damage to 9.5 day rat embryos in vivo of 41 °C (+2.5 °C over controls), 42 °C (+3.5 °C) for 10 min and 43 °C (+4.5 °C) for less than 2 min.

Heat shock genes and early brain development

The initiation of the early CNS and brain begins at neural tube closure, at approximately 3 weeks of human pregnancy. This is one of the most sensitive and critical periods of mammalian development. Interruption to neural plate development and induction of the neuroectoderm (progenitor cells of the brain) results in major craniofacial defects and mental retardation. Neural tube closure is one of the most critical stages of early embryogenesis. The pattern formation of the mammalian brain is one of the least known processes in embryology and is extremely sensitive to heat shock [60]. The structure of the early mammalian craniofacial region and CNS is predetermined in the neural plate during gastrulation and interruption to this process leads to dramatic craniofacial defects (fig. 1). The development of the the vertebrate CNS and heart begins with the induction of the neural plate on the dorsal surface of the embryo near the completion of gastrulation. Differentiating neuroectoderm exposed to heat shock causes rapid cell cycle changes resulting in either thermotolerance or cell death and major craniofacial defects particularly of the forebrain, heart and eye. The apical region of the anterior neuropore appears to be the region most effected and related to the development of

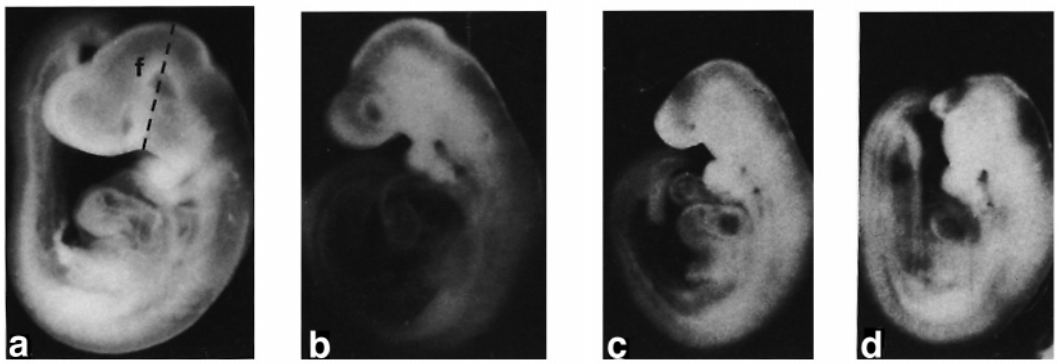

Figure 1. 11.5 day rat embryos after a 48 h in vitro culture following various heat shock exposures to the neural plate on day 9.5. *a)* 42 °C for 7 min; *b)* 42 °C for 10 min (heat shock response); *c)* 42 °C for 10 min then 38.5 °C for 1 h followed by a 43 °C for 7.5 min (acquired thermotolerance). *d)* 15 min at 42 °C, *d)* 43 °C for 7.5 min (teratogenic), note complete absence of developed forebrain and eye. Embryos at neural plate stage exposed to 42 °C and 42/43 °C showed a 2–4 h growth delay but apparent normal development after 48 h. Embryos exposed to 43 °C showed increasing severity of damage, particularly to the head and heart.

the perspective forebrain, midbrain, hindbrain and spinal cord. How the *hsp* and HSPs are activated and relate tocell death and cell fate influencing neuronal and glial cell induction and formation remains unknown.

The heat shock genes (*hsp*) and heat shock proteins (HSP)

The formation of the forebrain Walsh and colleagues at Sydney extended the work by Cockroft and New, by using rat embryos in culture to study induced thermotolerance and the heat shock response and its effects on post-implantation embryonic development.

Elevated temperatures provoke a response which is basically similar in plants and animals whether multicellular or unicellular, embryonic or mature and (in animals) vertebrate or invertebrate [61–63]. This indicates the fundamental importance of the adaptive response, known generally as the heat shock response. As it can also be elicited by certain other stressful agents including hypoxia, deprivation of glucose, heavy metals, ethanol, metabolic poisons, protein denaturants, ultrasound, it is also termed the stress response. It is characterised generally by the rapid inhibition of normal protein synthesis and the concurrent rapid, coordinated synthesis of a group known collectively as heat shock proteins (HSP) or stress proteins. The response is brought about by the activation of the heat shock genes (*hsp*), when the organism is exposed to a sufficient dose of heat or certain other stresses. After the induction of the response, the organism is more resistant to otherwise lethal or seriously damaging exposures to that, and to some other stressors. Ritossa [64] first described the onset of this reaction in heated *Drosophila* pupae in which gene activity was noted in the form of puffing patterns in their salivary gland polytene chromosomes. The subsequent research which led to the identification of the protective response used heat as the stress, hence the terms, the heat shock response and heat shock proteins.

Two major groups of stress proteins have been identified, based on the response to different stresses, the HSP and the glucose regulated proteins (GRP). The HSPs are enhanced by heat shock or hyperthermia and also by exposure to heavy metals, alcohol, metabolic inhibitors and protein denaturants. The GRP are synthesised in response to deprivation of glucose, oxygen and substances which disturb protein transport and calcium metabolism. Within the two major groups, some distinct families are recognised, based on their molecular weights (kD). This categorisation of HSP by size also relates generally to their functions. The HSP families which have been studied in embryonic development are HSP90 (a family with a molecular weight about 90 kD), HSP70, HSP47, HSP27 and ubiquitin which is the smallest HSP. Most HSP have two or more genes, an inducible copy which is induced by heat or other stresses and a constitutively expressed cognate (HSC) which is involved in normal cellular activities including chaperone functions.

Many stress proteins are also molecular chaperones, which facilitate polypeptide transport and the folding of newly synthesised proteins into their functional configurations but without becoming incorporated into the final product. Chaperone proteins protect newly synthesised proteins against inappropriate folding and against interactions with uncovered active surfaces on other proteins to form functionless aggregates. Newly induced HSP bind to thermally sensitive proteins in the cytoplasm while others translocate to the nucleus binding to, and protecting nuclear protein complexes [62]. The HSP with chaperone functions include HSP90, HSP70, HSP47 and the small HSP27 families. It appears that heat inducible HSP and some HSC also protect and

reconstitute heat damaged proteins by binding to uncovered, active sites of partially unfolded proteins, protecting them against binding randomly to similar sites on other damaged proteins. Reactivation of the protein being made possible by an ATPase releasing reaction to allow the disengagement of the HSP and HSC allowing the correct tertiary structure of the rescued protein to be completed.

HSP90: The HSP90 family is present constitutively in relatively large amounts in the cytosol of normal unstressed cells. Its function is uncertain but it might act as a molecular chaperone. After heat stress, it is only moderately induced. It has transient interactions with steroid receptors, actin and tubulin and is associated with microtubules of interphase cells and the spindle of mitotic cells. GRP94, a glucose regulated protein, is a member of this family. In differentiating neuroectoderm it was noticed that HSP90 was highly expressed in the G0 phase or resting phase of the cell cycle. Following severe heat shock HSP90 was reduced possibly allowing cells to progress through to G1 + S phase, suggesting that HSP90 maybe required to maintain cells G0 phase [65].

HSP70: The HSP70 family has several members with different functions in differentiating neuroectoderm. The constitutive HSC73 (a heat shock cognate) is normally present at high levels during neural tube closure and neuroectoderm differentiation and proliferation. It was identified in the cytosol and nucleus and is moderately induced by heat. Under normal conditions HSP71 is present in very small quantities, it is strongly induced by heat, being found in the cytosol and transmigrating into the nucleus following stress in neuroectoderm and mesoderm cells. HSP71 binds to proteins synthesised under stressed conditions and to damaged proteins.

The synthesis of the HSP71 during normal cell cycle progression was identified in G2 + M phase of early neuroectoderm cells during brain development. The inducible HSP71 was tightly regulated in the cell cycle and only appeared at very late S phase and G2 + M. The possible function of HSP71 in normal cellular division and proliferation is unclear in the embryo but the cell is under considerable natural stress at this stage of its division. Heat shock induces the synthesis of HSP71 in all stages of S phase and its appearance appears to be associated with the period of the cell in its state of acquired thermotolerance. Once the cell has progressed through G2 + M stage returning to G0 the HSP71 appears to have been degraded and the cell appears to be highly susceptible to heat shock

HSP47: HSP47 has been identified recently as a stress protein [66–70]. It is a member of the serpin group, situated in the endoplasmic reticulum and has been confirmed to bind to types I–V collagens in vitro. At neurulation it is present constitutively in both neuroectoderm and mesoderm cells and is strongly induced by heat. During neural plate induction HSP47 is strongly associated with collagen IV synthesis and in the formation of the basement membrane and extracellular matrix. HSP47 was also identified with neural crest cell migration especially from the cervical 5–8 region that is responsible for the establishment of the greater vessels of the heart [71].

HSP27: The low molecular weight family HSP27 is present constitutively and is strongly induced by heat, and is a molecular chaperone, particularly of the actin cytoskeleton. Ubiquitin is the smallest member having a molecular weight of about 8000 and is induced by heat and also binds to and tags damaged proteins for degra-

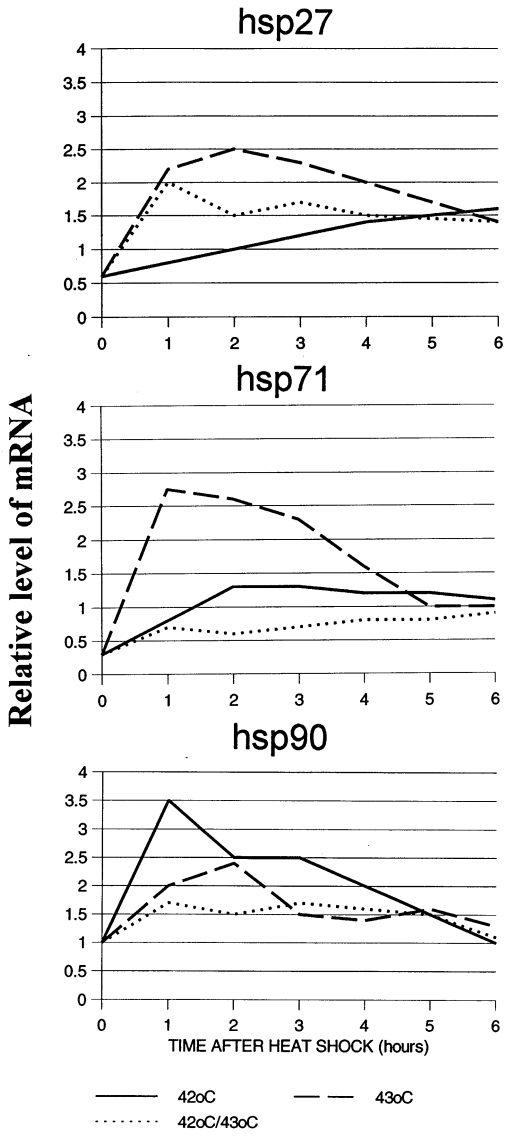

Figure 2. Changes in hsp27, 71 and 90 mRNA during neural plate induction at 9.5 day in cultured rat embryos after exposure to a 42, 42/43 and 43 °C heat shock using Northern and dot blot analysis. The response to a 42 °C followed within 1 h by 43 °C showed down regulation of hsp71 and 27 compared with 43 °C alone. The expression of hsp90 mRNA was proportional to the total dose of heat given.

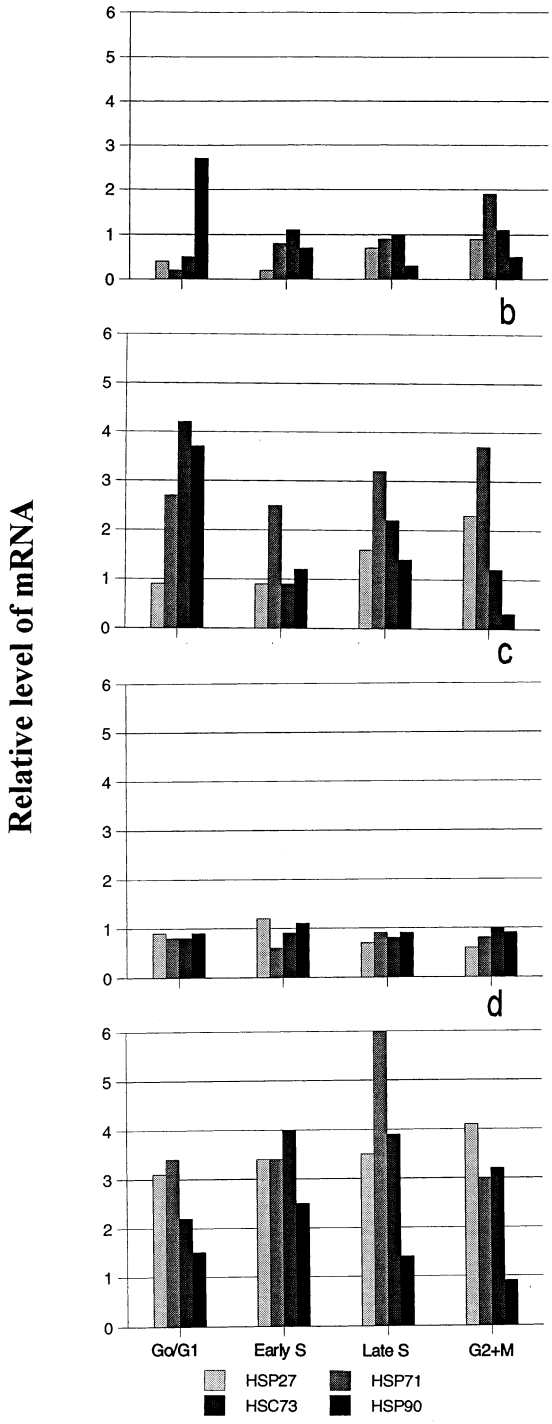

Figure 3. The rapid cell cycle changes and regulation in hsp90, 71 and 27 and hsc73 mRNA in neural plates at 9.5 day cultured rat embryos during G0/G1, early S phase, late S phase and G2 + M; *(a)* Control transcription rates during the cell cycle; *(b)* 42 °C for 10 min. *(c)* 42 °C for 10 min followed by 38.5 °C for 1 h, then 43 °C for 7 min; *d)* 43 °C for 7 min. Embryos were processed by flow cytometry, dot blot and Northern analysis at the 1 h time point following heat shock. After 42 °C, a fourfold increase of all hsp mRNA occurred and at 43 °C all hsp were overexpressed. After 42/43 °C, there was a general down regulation of all the *hsp* genes.

dation. It is present constitutively associated with histones in the chromatin of the nucleus.

The heat shock response in neural plate

Walsh et al. [5, 10] used 9.5 day (early head fold, pre-somite) rat embryos, which were cultured using a modification of the method of New et al. [72] at 38.5 °C throughout the 2 days of the culture period (controls). After 2.5 h at 38.5 °C some were exposed to various regimes of hyperthermia for up to 80 min and then returned to the cabinet at 38.5 °C for the remainder of the 2 day culture period. In dose-response experiments, exposure to 43 or 43.5 °C for as little as 0.5 min caused significant deficits of protein accumulation measured on day 11.5. Microphthalmia, neural tube defects and reduced forebrain were caused by the lowest doses. A temperature of 43.5 °C (an elevation of 5 °C) for 2.5 min, an elevation of 4.5 °C for 7.5 min, 4 °C for 10 min or 3.5 °C for 40 min caused one or more defects, showing that as the temperature is increased, the time required to cause defects is reduced logarithmically. Also, as time at a given temperature increases, the severity of defects is increased (fig. 1). These findings match those of Germain et al. [9] who used similar temperature and duration combinations in rat embryos in vivo.

These experiments also showed that 42 °C for 10 min resulted in normal, but slightly developmentally retarded embryos when examined at 11.5 days. This exposure also conferred a significant degree of protection against a subsequent exposure to 43 °C for 7.5 min, which otherwise causes severe malformations. The acquisition of thermotolerance after the 42 °C exposure required a recovery period of at least 15 min at 38.5 °C. Thermotolerance was not acquired during continuous exposure to 42 °C for 20–40 min, which caused severe head defects. The acquired thermoprotection persisted for at least 8 h and during this period, general protein synthesis was reduced while synthesis of proteins of 71–73 kD and 90 kD increased (fig. 2). Control embryos contained high levels of 73 kD and 90 kD proteins. Based on their molecular weights and appearance during the heat shock response, these proteins were assumed to be members of the HSP70 and 90 families.

Following these initial studies, the heat shock response was examined in more detail again using 9.5 day rat embryos given one of three types of exposure: *1)* 42 °C for 10 min which does not cause defects, but following an adequate recovery period at 38.5 °C, it confers thermoprotection against a subsequent damaging exposure, *2)* 43 °C for 7.5 min which causes severe developmental damage, or *3)* 42 °C for 10 min, followed by recovery at 38.5 °C for 1 h, then 43 °C for 7.5 min. These embryos show no developmental damage at 11.5 days (fig. 3).

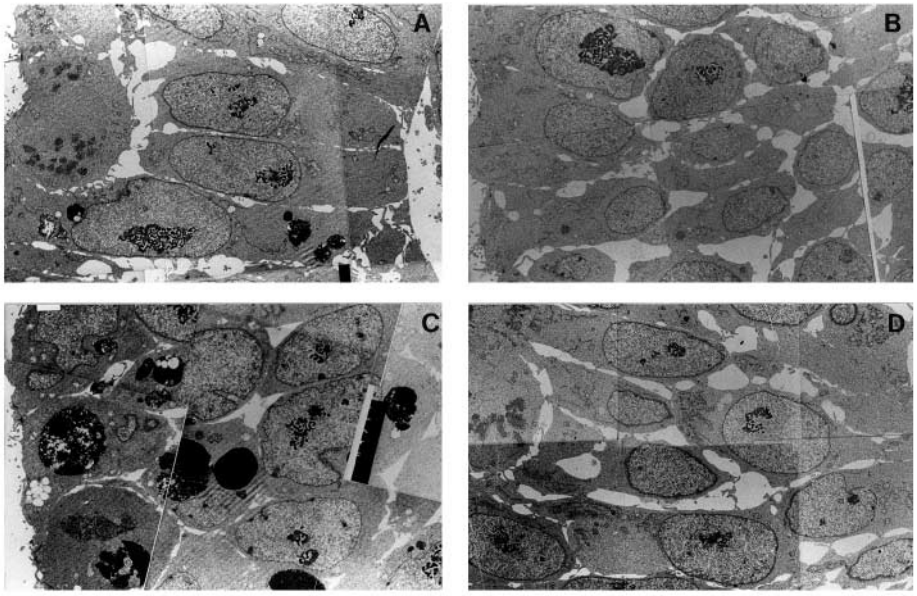

Figure 4. Cell death observed in the neural plate of 9.5 day cultured rat embryo following exposure to heat. The neural plate was cut from 6u transverse plastic section to show the neuroectoderm layer, ventricular surface (left open arrow) and basement membrane (right black arrow). *A)* neuoectoderm 4 h after a heat shock exposure at 43 °C for 7 min; note neuroectoderm cell death. *B)* neuroectoderm 4 h after exposure to 42 °C for 10 min, there is little evidence of cell death and DNA clumping in the neuroectoderm *C)* neuroectoderm 6 h after exposure to 43 °C for 7 min, showing extensive cell death and disruption to the neuroectoderm layer *D)* neuroectoderm 6 h after exposure to a 42 °C for 10 min followed by a further heat shock at 43 °C for 7 min showing acquired thermotolerance compared to 43 °C alone (panel C).

In these studies [57, 60, 65, 74], HSP90, HSC73 and HSP23-27 were found in normally developing control 9.5 day rat embryos but HSP71 was not detected. At 1.5 h after the damaging 43 °C exposure, there was a reduction in total protein synthesis of over 30% and at 2–3 h a four fold increase in the synthesis of HSP71/73 and 90. The levels of synthesis of total protein and HSP71/73 and 90 had returned to normal by 6–8 h (fig. 4).

Recently, the expression of HSP47 has been studied by Li et al. [71]. Using immunohistochemistry in 7.5 day rat embryos, it was found to be confined to parietal endoderm cells. At 8.5 days it was present in ectoderm, mesoderm and endoderm. It was widespread on day 9.5 appearing generally through the ectoderm, mesoderm and endoderm of the embryo, its allantois and yolk sac. After 9.5 days it was found in more regionally specific areas including the brain, branchial arches, heart and somites and except for ectodermal tissues, coinciding generally with the distribution patterns of collagens I and IV. Although HSP47 was found in the ectoderm at day 9.5, no collagen could be identified in it until after the commencement of neural tube closure.

Changes during the heat shock response in levels of hsp27, 71 and 90 mRNA were studied using Northern (fig. 5) and dot blot analysis. Northern analysis could not detect 71 kD mRNA in control embryos but it was present in small quantities at 60 min after the 42 °C exposure. It was detected within 20 min after exposure to 43 °C and reached a peak of about 8 fold over resting levels in 90 min declining to normal levels in 5 h. The levels in the 42/43 °C embryos were much less than the 43 °C group. HSC73 was present in all samples, in small amounts in controls and embryos exposed to 42 °C but in larger quantities in embryos after the exposures at 43 °C and 42/43 °C. HSP27 was also induced quickly by heat shock and after the 42 °C exposure rose to about a two fold level between 0.5–3 h falling to normal levels by 6 h. Much higher expression occurred after the 43 °C exposure and the 42/43 °C treatment resulted in levels lower than both the 42 and 43 °C responses. The response with hsp90 mRNA was different. Following 42/43 °C they were higher than those for 43 °C which in turn were higher than those for 42 °C, suggesting that the response for hsp90 mRNA is proportional for the total dose of heat.

Dot blot analyses after 42 °C showed a rapid activation of hsp71. Within 20 min, a 2–3 fold increase of mRNA was detected in the nucleus, increasing slightly over the next 60 min and then falling to control levels in 3–4 h. The levels in cytosol showed a similar response but returned to normal within 2.5 h. Following the 43 °C exposure, the nuclear mRNA also showed a 2.5 fold increase within 20 min, this level being maintained for over 4 h and falling to base levels. The cytosol fraction also showed a rapid increase to a 6 fold peak at 1.5 h, falling rapidly to normal levels by 5 h. The hsc73 mRNA showed about a 2 fold increase within 60 min. The thermotolerant embryos (42/43 °C) showed an intermediate response.

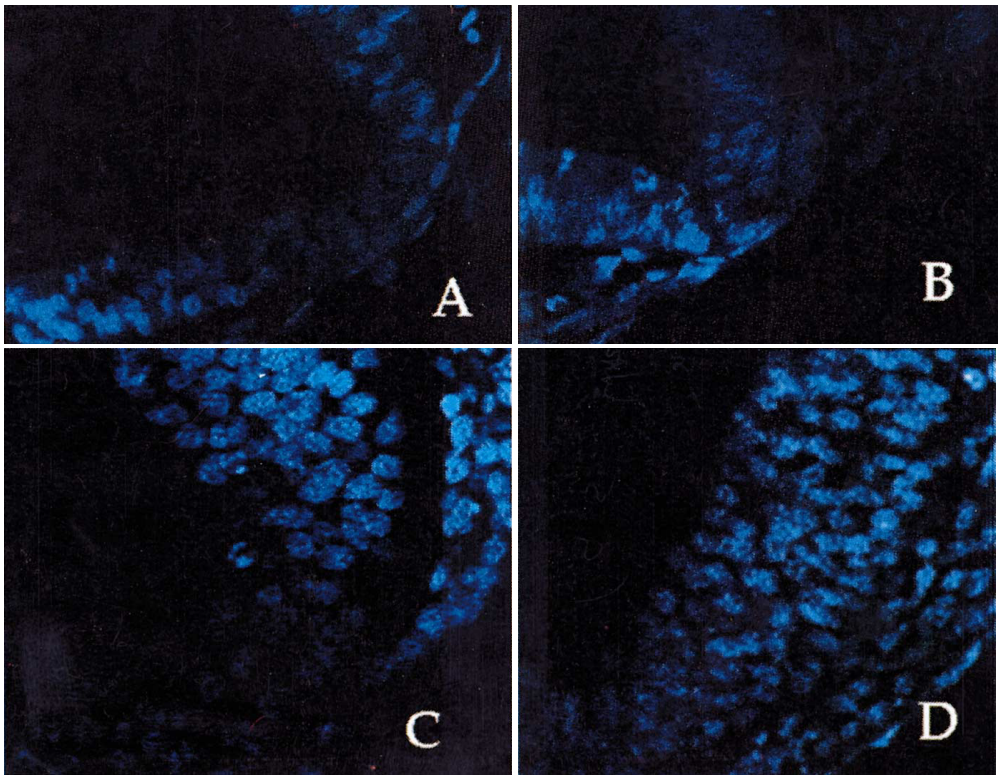

Figure 5. HSF1 (*A*, *C*) and HSF2 (*B*, *D*) in the anterior neuropore region of the neuroectoderm of 9.5 day cultured rat embryos, a cross section of the neural plate was scanned by Confocal microscopy using specific HSF antibodies 1 hr following a 42 °C for 10 min (*C*, *D*) heat shock with both HSFs appearing to concentrate in the neuroectoderm nucleus following heat shock at this stage.

In situ hybridisation studies of control 9.5 day embryos using hsp cDNA probes, showed constitutive expression of hsp71 only in the allantois and ectoplacental cone. In embryos given the 43 °C exposure, maximum hsp71 expression was at 90–120 min in the neurectoderm of the neural plate, its underlying mesoderm and at low levels in the endoderm. Expression was most marked around the anterior neuropore. In 10.5 and 11.5 day embryos given the 43 °C exposure, the overall hsp71 expression was reduced being greatest around the mid- and hindbrain areas.

The developmental expression and distribution of hsp47 mRNA was studied recently, using Northern blot analysis and whole mount in situ hybridisation [71]. With the exception of ectodermal structures, the expression of hsp47 mRNA coincided with the distribution patterns of collagens I and IV. Although hsp mRNA was expressed in the ectoderm before neural tube closure, collagen was not detected until after it commenced. It was expressed most strongly between days 9.5 and 11.5 and at lower levels at days 13.5 and 14.5 (fig. 6) and was found in ectoderm and endoderm of 8.5 day embryos and was widespread in the neural plate, yolk sac, allantois and chorion at 9.5 days. At 10. 5 days its distribution was confined to the brain, heart, branchial arches and somites.

The heat shock response in later stages of embryonic brain development

The heat shock response in later stage neuronal and glial development changes considerable depending on the proliferative capacity of the region [75]. Mirkes [53,

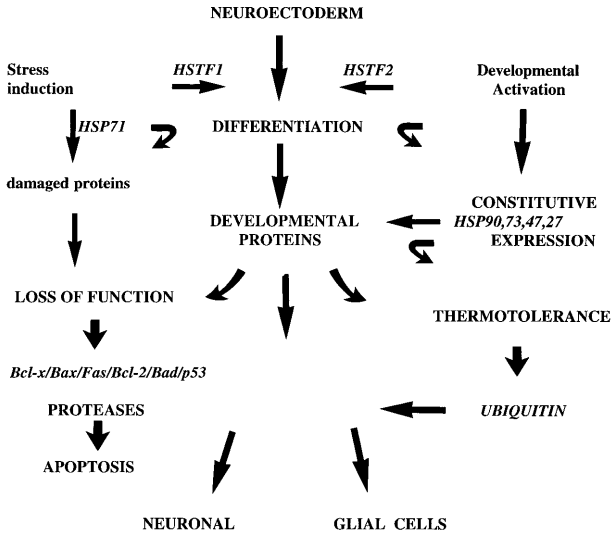

Figure 6. HSPs and HSFs in the differentiation and induction of the neuroectoderm in the developing neural plate.

76] used four treatment groups of day 10 (6–10 somite) rat embryos. Controls were cultured throughout at 37 °C. A second group was at 37 °C throughout except for 30 min at 42 °C, given 1 h after the culture started. A third group was at 37 °C throughout, with 43 °C for 30 min given at 2.5 h, while a fourth group received exposures of 42 and 43 °C as above, separated by 1 h at 37 °C. At examination on day 11, the 42 °C group did not differ from controls, the 43 °C embryos had a high mortality and level of defects, while the 42/43 °C embryos had partial protection against mortality and malformation.

Within 1 h, the 43 °C treatment induced the synthesis of HSP27, 71, 78 and 90. Synthesis of all induced proteins ceased between 3 and 9 h after exposure except HSP27 which ceased at 1–3 h. Usually, normal protein synthesis is curtailed during the heat shock response but in these experiments, the 43 °C embryos did not show such a reduction. After the 42 °C exposure, synthesis of HSP71 was induced and the combined treatment, except for the HSP27 induced all proteins at about half the level of the 43 °C treatment.

Fisher et al. [77] also used heat shock in day 10 rat embryos in vivo and in vitro to examine the role of the heat shock response in the genesis of defects of somite segmentation, resulting in vertebral and rib anomalies of the mid thoracic region [78]. After exposures in vivo to 42–42.5°C for 5 min or in vitro to 42–42.5 for 15 min, they found enhanced synthesis of a HSP71 for 1 to 4 h and of a 90 kD protein for 1 to 8 h. Transcription was required for its expression. The inducible HSP71 was identified by Western blot which accumulated and remained in the neuroectoderm 2–27 h after exposure. It was not detected in the somite mesoderm. There was a lag period of 18 h between accumulation of HSP71 and the appearance of abnormal segmentation and the severity of damage to somites was related to the dose of heat. The authors commented that the absence of heat shock response in the somite mesoderm might explain its sensitivity to heat. However, this argument cannot be applied to the damage caused by heat on day 9 to the neuroectoderm which accumulates large amounts of induced HSP before and during the period of abnormal neural plate development [10]. In studies on somite segmentation, analysis of the HSP 47 response might be rewarding.

The response in days 9 (pre-somite), 10 (6–10 somite), 11 (21–25 somite) and 12 (31–35 somite) rat embryos were compared following an extreme exposure to a severe heat shock of 43 °C for 15–60 min and returned to 37 °C for 1 h before processing [79]. The HSP response was analysed by two-dimensional gel electrophoresis and mRNA by Northern blot analysis. Day 9 and 10 embryos synthesised HSP 27, 71,73 90 essentially the same sets of HSPs. The response on day 11 was limited to HSP27, 71 and on day 12 none were found. Northern blot analysis of hsp70 mRNA showed the response varied with the stage of development with day 9 embryos showing the greatest response.

It is known that agents other than heat shock can elicit a heat shock response. Sodium arsenite added to the culture medium to a level of 50 μM for a 2.5 or 5 h exposure induces HSPs similiar to the heat shock exposure of 15 min at 43 °C and a monoclonal antibody to the inducible HSP72 identified it in embryos exposed to hyperthermia or sodium arsenite [80]. Although the response was similar, the defects caused by the two treatments were different, heat shock causing more severe damage to the developing prosencephalon, rhombencephalon and eyes.

Developmental regulation of HSP expression

As some constitutive HSP are present at certain embryonic stages, it can be inferred that they play a role in normal development. The 70 kD family are the first to be expressed constitutively at the 2 cell stage and HSC70 is also at high levels at the 8 cell stage. In the mouse, very active constitutive expression of members of the 90 kD, 70 kD and 60 kD families occurs at the 8 cell stage, with the heat inducible HSP86 and HSCs84 and 70 being most prominent. During subsequent development, this level of synthesis is maintained. These constitutive stress proteins form a relatively large proportion of the total protein content of the neuroectoderm at day 8, the stage of neural induction in the mouse [81] and in the rat, at days 9.5–11.5, the stages of neural induction and major organogenesis [73]. Transcriptional activation is mediated by a heat shock transcriptional factors (HSF) which is present in the cytoplasm and nucleus of unstressed embryonic cells. At some stages of development and in response to heat or other stresses, the transcription factor accumulates in the nucleus, binding to DNA at a site known as the heat shock element [82].

The inducible HSP are not found normally in embryonic tissues except in small amounts as they are not transcribed, but their synthesis can be induced by heat at certain stages of development. The heat inducible hsp70 can be induced for a brief period after cleavage to form a 2 cell embryo and is strongly induced after the blastocyst stage [83, 84]. It is not inducible in mouse and rabbit embryos between the 2 cell and blastocyst stages. The work outlined above indicates that the response can be induced strongly during the major stage of organogenesis in the rat (days 9–12). With the known functions of the HSP, and the evidence that the onset, and the duration of inducibility of Hsp, coincides with onset and duration of the most critical stage of organogenesis, it appears that a function of the heat shock response could be to provide protection against embryonic damage by heat and other stresses at vulnerable stages of development.

Cell cycle regulation

Cell proliferation and cell fate specification are under strict spatiotemporal control in the developing neural plate and early brain formation. During mammalian development of the brain precursor cells undergo characteristic patterns of cell division before commitment to specific fates. Such invariant divisions may result from dependence of cell fate on cell lineage and a precise pattern of divisions may be necessary to achieve the required distribution of cytoplasmic determinants. However, in some cases, cell division is tightly controlled even though cells adopt fates independently of their lineage history.

Using flow cytometry, Walsh and Morris [85] and Walsh et al. [74] studied cell cycle changes and regulation and expression levels of hsp27, 71, 73 and 90 mRNA at various stages of the neurectodermal cell generation cycle and the effects of the various heat shock regimes (fig. 3). The analysis was done at 1 h after heat treatment. In control 9.5 day rat neurectoderm, there was a steady, even expression of transcripts hsc73 throughout the cycle and a high expression of hsp90 at Go-G1 and hsp71 at G2-M. With a 42 °C for 10 min, the levels of all mRNA were elevated in Go-G1 and late S phases, with high levels of hsc73 and hsp90 at Go-G1, while hsp27 and 71 transcripts were elevated above control levels at all phases, particularly at late S and G2-M. With 43 °C for 7.5 min, hsp27 expression was greater throughout the cycle than for any other treatment, hsp71 was strongly expressed in late S and hsc73 was elevated in S and G2-M phases. It could be argued that the different levels of expression of the hsps at the various phases might reflect a response to different levels of damage to cell constituents but this proposal has not been tested. Some weight is given to this interpretation by the response following the combined, non teratogenic treatment of 42 °C followed by 43°C in which there is a general down regulation of expression of all mRNA hsp and HSP synthesis compared with the expression after either single treatment.

Apoptosis following heat shock

In the studies by Cockroft and New [29], histological examination of embryos cultured at 40.5 °C and 41 °C showed wide spread cell death, particularly in the brain and spinal cord. In embryos cultured at 41 °C parts of the nervous system were entirely necrotic. Neuroepithelial cell death was also the prominent feature of the pathological findings in heated guinea pigs [86–88], mouse [37] and rat embryos in vivo [25, 89] and in vitro [10, 60, 65, 76] and appears to be the most important basic mechanism underlying the neural defects caused by heat. Cell death, usually of less severity, is also observed in mesodermal cells, but is infrequent in endodermal cells. In guinea pigs, mitotic cells of the 21 day embryonic neuroepithelium are the most sensitive, abnormally clumped chromosomes being found immediately after a 45 min spike elevation of approximately 2 °C. Also after 4–8 h, cells presumed to be in S-phase and some mesoderm cells showed apoptotic cell death. Heat damage to cells in S-phase requires an elevation of at least 3–3.5 °C. During the 4–8 h period, normal mitotic activity ceases and this period of inhibition is followed by a synchronised burst of mitotic activity [86–88]. The damaged M and S phase cells break up and are removed within a few to 24 h. Walsh et al. [60, 65] showed in 9.5 day cultured rat embryos, that 42 °C (+3.5 °C) for 10 min caused death of a small number of cells in the G2 or mitotic phases of the cell cycle at 5 h and also at 12–15 h after exposure, possibly after a division had occurred. No developmental abnormalities result from this dose of heat. However, a teratogenic exposure of 43 °C (+4.5 °C) for 7.5 min, was followed at 3–5 h by very extensive apoptotic cell death in the neuroectoderm particularly in the ridges of the neural folds [71]. This exposure in vivo causes microphthalmia in 100% of surviving embryos [9]. In their flow cytometry studies, Walsh and Morris [85] also found that the heat stressed neurectodermal cells caused a partial synchronisation of the generation cycle by causing cells to accumulate for 1–2 h at the G1-S boundaries after treatments of 42, 43 or 42/43 °C. Following treatments of 42 or 42/43 °C there was also a barrier to cell progression at the S-G2 boundary. Activation and enhanced transcription and synthesis of Bcl-2 was observed at early neural plate stage, after exposure to heat shock and the respective cell cycle changes seen both induction of the positive controlled Bcl-x and Bax apoptotic pathways were also observed in neuroectoderm.

The apoptotic cell death in embryos damaged by heat at neurulation should not be confused with the apoptosis which determines the shape of a structure or deletes supernumerary cells as a normal embryological mechanism [90]. At the time of neurulation, very few apoptotic cells are normally found and never in the very large numbers which follow a damaging exposure to heat. It has been suggested that once cells are committed to commence a division cycle, apoptosis is initiated and when the cell reaches check points with acceptable progression in the cell cycle it passes to the next phase [91]. Sufficient heat damage to the functional proteins of dividing embryonic cells might well prevent the successful completion of the immediate or the subsequent cycle and allow the apoptotic program to be implemented.

Discussion and conclusions

Heats shock or hyperthermia is a recognised teratogen in animals and there is strong evidence that it also

causes significant damage to human embryos. Studies with induced heat shock in pregnant animals and the defects caused were dependant on the susceptible stages of development and threshold doses of heat exposure. The in vivo experiments lacked precision because of variability of embryonic development at a given conceptual age, varying maternal responses to agents causing temperature elevations, the difficulty in measuring embryonic temperature and the possibility that defects were caused by toxic changes in maternal metabolism. These variables were eliminated by the use of postimplantation whole rat and mouse embryo cultures, which were exposed to various doses of heat at closely defined stages of development. The studies showed that heat acts directly on embryos and that elevations of 2 °C and greater sustained over early rat organogenesis cause defects mainly by causing apoptotic cell death especially in the developing central nervous system.

A moderate, nondamaging exposure is followed within 15 min by protection for up to 8 h against a more severe and otherwise teratogenic exposure. The protective heat shock response is accompanied by a reduction of normal protein synthesis and concurrent synthesis of heat shock proteins (HSP 90, 71, 47, 27). Most HSP in these families are also present constitutively in embryos (HSC73), probably having important roles in protecting newly synthesised proteins from aggregation and facilitating folding into their normal tertiary functional configurations. The appearance of induced HSP and hsp mRNA at known sites of thermal damage suggests a protective role. Heat induced cell death by apoptosis is a feature of teratogenic damage to the developing brain. Apoptosis could be a by-product of a damaging heat shock exposure because of the rapid interruption to the normal gene program during organogenesis, the induction of the HSPs and survival being achieved at the expense of normal development.

The information yielded from the in vivo and in vitro studies allows some conclusions and speculations to be made. A fundamental question is, what makes some embryonic cells extremely sensitive to damage by heat and other agents? Embryos can tolerate variable doses of heat shock depending mainly on the stage of development. The most clearly defined threshold elevation of temperature causing defects in vivo is 2–2.5 °C over a total heating and cooling period of about 80 min in 21 day guinea pig embryos [40] and for 60 min in 9.5 day rat embryos [9]. A similar threshold temperature elevation has been shown for 9.5 day rat embryos in culture [5, 28]. It has been suggested that the threshold of 2–2.5 °C exists because of the presence in cells of constitutive, heat shock 90, 70, 47 and 27 chaperone proteins which can protect against denaturation [2] so the threshold might represent a quantitative measure of the denaturation required to titrate out the constitutive proteins. It has long been known that heat denatures proteins [92] and even at normal body temperatures (37–40 °C), proteins are denatured. At 37 °C, this results in an estimated loss of over 0.2% of cells per hour [93].

There is ample evidence that a strongly protective heat shock response need not be teratogenic, but there is equally strong evidence that a teratogenic dose of heat is associated with highly elevated hsp mRNA and HSP and that cell death is a prominent feature of the damage to the nervous system [73]. Perhaps there is a threshold level for the response, above which developmental program cannot be rescued, and below which apparently complete recovery occurs. The basic question is whether defects occur because of the activity of the heat shock response or because of its failure to protect or possible ability to replace and regenerate.

Close examination of the stages at which most damage is caused to embryos indicates that it is at the inductive stage for the formation of an organ especially the brain. The stage of active cellular proliferation to form the organ is less susceptible to damage by the same amount of heat and after formation, the organ becomes relatively resistant. It may be that the heat shock response is greatest in organs most susceptible to heat; that the heat shock response can fail to protect embryos during the inductive phase of organogenesis and that cells which die are concentrated in the immediate region being induced. It is also possible that during the induction of an organ, the protective, constitutive heat shock proteins are largely requisitioned by heat susceptible, newly synthesised proteins and are unavailable as a reserve. Another possibility is that the cells partially induced for organogenesis cannot mount a heat shock response and survive.

The most perplexing question is why the heat damaged embryo is unable to replicate more cells to make up the mass required to form a normal organ? In many instances it appears that one further division by the replicating cell population would easily make up the deficit. For instance, after heat damage to an embryo at neural tube closure, the compensatory proliferation of only a minor proportion of the total cell population could provide the mass required for closure.

The first rapid phase of neurogenesis of the fetal brain ceases at a specific day whether the target growth has been achieved or not [59] and a possible explanation for a 'timed'termination of proliferation is that, at induction of an organ, the induced cells are programmed to a finite number of divisions. In this model, cells lost by apoptosis following heat exposure would not be replaced. We are still working on the resolution of these uncertainties using embryos in culture.

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