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Effects of heat shock on survival, proliferation and differentiation of mouse neural stem cells

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ABSTRACT

Hyperthermia during pregnancy is a significant cause of reproductive problems ranging from abortion to congenital defects of the central nervous system (CNS), including neural tube defects and microcephaly. Neural stem cells (NSCs) can proliferate and differentiate into neurons and glia, playing a key role in the formation of the CNS. Here, we examined the effects of heat shock on homogeneous proliferating NSCs derived from mouse embryonic stem cells. After heat shock at 42 °C for 20 min, the proliferating NSCs continued to proliferate, although subtle changes were observed in gene expression and cell survival and proliferation. In contrast, heat shock at 43 °C caused a variety of responses: the up-regulation of genes encoding heat shock proteins (HSP), induction of apoptosis, temporal inhibition of cell proliferation and retardation of differentiation. Finally, effects of heat shock at 44 °C were severe, with almost all cells disappearing and the remaining cells losing the capacity to proliferate and differentiate. These temperature-dependent effects of heat shock on NSCs may be valuable in elucidating the mechanisms by which hyperthermia during pregnancy causes various reproductive problems.

Abbreviations: ESC, embryonic stem cell; NSC, neural stem cell; NSS, neural stem sphere; CNS, central nervous system; NTD, neural tube defect; ED, embryonic day; ACM, astrocyte-conditioned medium; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; PM, proliferation medium; DM, differentiation medium; MAP2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; HSP, heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; BSA, bovine serum albumin; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling.

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1. Introduction

Hyperthermia, defined as a body temperature higher than normal, can be induced by fevers, environmental exposure to heat sources and some drugs. Hyperthermia causes heat shock stress in cells, inducing the expression of genes and proteins encoded by heat shock protein (HSP) genes (reviewed by Arya et al., 2007). This may result in damage to cells, with severe hyperthermia causing cell death, as well as of damage to tissues, organs and the body. Hyperthermia during pregnancy is a significant cause of reproductive problems in many animals, including mice, rats, guinea pigs, lambs and monkeys (reviewed by Edwards, 2006), ranging from embryonic death and abortion to teratogenically induced anomalies. These effects are highly dependent on the extent of temperature elevation, its duration and the timing of exposure. Higher temperatures and/or longer duration are more likely to cause abortions, whereas lower temperatures and/or shorter duration are more likely to cause embryonic death and resorption. Moreover, birth defects may be induced if exposure to hyperthermia occurs at critical stages of development.

Malformations induced experimentally after exposure of animals to hyperthermia involve many organs and structures. However, malformations affecting the central nervous system (CNS) are most common and include neural tube defects (NTDs) such as anencephaly or encephalocele, microphthalmia, microcephaly, cranial nerve defects, behavioral abnormalities, disturbances of muscle tone, talipes, arthrogryposis multiples congenia, and reduced learning capacity (reviewed by Edwards et al., 1995, 1997). Experimental studies in guinea pigs have demonstrated that congenital defects in the CNS depend on the timing of hyperthermia relative to stage of development (Edwards, 2006). Most NTDs are caused by hyperthermia on embryonic days (EDs) 13–14, during the stage of neural tube formation (Cawdell-Smith et al., 1992), whereas microcephaly is caused by hyperthermia on EDs 20–23, during the early neurogenic phase (Edwards et al., 1984). Toward the end of major neurogenesis and at the onset of gliogenic phase (EDs 39–45) hyperthermia causes microcephaly associated with learning and behavioral abnormalities (Edwards, 2006). The relationships between types of CNS defects and the timing of hyperthermia have also been demonstrated in other species. NTDs were induced by hyperthermia during the stage of neural tube formation, on ED 8.5 in mice (Shiota, 1988) and ED 9.5 in rats (Walsh et al., 1987). Additionally, microcephaly was induced by hyperthermia during later stages of development of the CNS, on EDs 12–15 in mice (Shiota and Kayamura, 1989) and EDs 13–14 in rats (Harding and Edwards, 1993). Clinical observations and epidemiological studies in humans have indicated that hyperthermia is a teratogen, with NTDs and microcephaly being the most common and serious defects (Edwards et al., 1997). Moreover, hyperthermia resulting from use of a hot tub or spa early during pregnancy was found to increase the risks of NTDs and spontaneous abortions in humans (Chambers, 2006; Edwards et al., 1997).

During nervous system development, neural stem cells (NSCs) can self-renew by symmetric divisions and then differentiate into neurons, astrocytes and oligodendrocytes via asymmetric divisions, playing a key role in the formation of nervous system (Temple, 2001; Merkle and Alvarez-Buylla, 2006). Harmful effects of hyperthermia on NSCs have been reported in experimental animals in vivo and in cultured embryos (Edwards et al., 1974; Edwards, 2006). In guinea pigs, hyperthermia at ED 21 caused a temporary cessation of proliferation and induced death of the proliferating neuroepithelial cells, viz. NSCs (Wanner et al., 1976). In vivo exposure of mouse embryos on ED 8.5 or ED 9.5 to hyperthermia was also reported to induce a temporary cessation of proliferation and death of NSCs (Shiota, 1988). Hyperthermia was found to act directly on ED 9.5 rat embryos in culture, causing apoptotic cell death of neuroepithelial cells (Edwards et al., 1997). Taken together, these findings suggest that hyperthermia causes malformation of the nervous system through harmful effects on NSCs. Therefore, it is very important to elucidate the direct effects of hyperthermia on NSCs. We have previously reported that a large number of homogeneous NSCs can be directly produced from embryonic stem cells (ESCs) by a simple method, the neural stem sphere (NSS) method (Nakayama et al., 2003; Nakayama and Inoue, 2006). NSCs prepared by this method can be expanded in the presence of fibroblast growth factor-2 (FGF-2) (Nakayama et al., 2004) and differentiated into neurons and glia (Nakayama et al., 2003, 2006; Otsu et al., 2011). We have therefore analyzed the effects of heat shock on the survival, proliferation and differentiation of homogeneous NSCs prepared from mouse ESCs in vitro and found that exposure of NSCs to hyperthermia induced apoptosis and suppressed proliferation and differentiation.

2. Materials and methods

2.1. Preparation of NSCs by the NSS method and culture

Mouse NSCs were prepared from undifferentiated mouse ESCs by the NSS method, as described (Nakayama et al., 2003; Nakayama and Inoue, 2006). Briefly, colonies of ESCs were cultured under free-floating conditions in astrocyte-conditioned medium to induce their uni-directional differentiation into neural cells, resulting in NSSs, visible as floating spheres containing NSCs. These NSSs were plated onto matrigel-coated dishes and encouraged to proliferate by culture in neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 2% B-27 (Invitrogen) and 20 ng/ml FGF-2 (R&D Systems, Minneapolis, MN) (proliferation medium, PM) (Nakayama et al., 2004; Isono et al., 2012). NSCs that had migrated from the adherent NSSs to surrounding areas were harvested, and homogeneous NSCs were allowed to proliferate exponentially on matrigel-coated dishes in PM. To induce NSC differentiation, the medium was replaced by DMEM/F-12 (DF) (Sigma–Aldrich, St. Louis, MO) medium supplemented with N-2 (Invitrogen) (differentiation medium, DM) (Isono et al., 2012).

2.2. Exposure to heat shock

Proliferating NSCs were harvested by trypsin treatment, and the cells were suspended in PM. Each cell suspension was added to a 19 volumes of PM in 15 ml centrifuge tubes (Sumitomo Bakelite, Tokyo, Japan), which had been pre-heated in thermostat water bathes (Thermo minder; TAITEC, Saitama, Japan) to 37 °C (control), 40 °C, 42 °C, 43 °C and 44 °C. The tubes were incubated for 20 min at these temperatures; the temperature of the medium in these tubes remained stable from the start to the end of heat shock. The cells were subsequently seeded at a density of $2 \times 10^3/cm^2$ on matrigel-coated 35 mm dishes (BD Falcon; Becton Dickinson, Franklin Lakes, NJ) and cultured in PM for 4 days, with culture medium changed on day 1 and 3. In some experiments, NSCs cultured on dishes were exposed to heat shock in situ by incubating the dishes, which were tightly sealed by parafilm, in thermostat water bathes for 20 min. In these experiments, the temperature of the medium in the dishes reached the set temperature within 7 min. After heat shock, NSCs were cultured as described above. The numbers of cells on dishes were determined from phase-contrast images of the cells using an inverted microscope (Eclipse TE300; Nikon, Tokyo, Japan). Five or ten images per dish were obtained every 24 h, and the numbers of cells were counted.