CHAPTER 8

The effect of induced hyperthermia on the immune system

Annette Dieing1,* , Olaf Ahlers2, Bert Hildebrandt3, Thoralf Kerner2, Ingo Tamm3, Kurt Possinger1 and Peter Wust4

1Department of Oncology and Hematology, Charité Campus Mitte, University Medicine Berlin, Germany, Schumannstrasse 20/21, D-10117 Berlin, Germany
2Department of Anesthesiology and Intensive Care Medicine, Charité Medical School, University Medicine Berlin, Campus Virchow Clinic, D-13353 Berlin, Germany
3Medical Clinic, Department of Hematology and Oncology, Charité Medical School, University Medicine Berlin, Campus Virchow Clinic, D-13353 Berlin, Germany
4Department of Radiation Medicine, Charité Medical School, University Medicine Berlin, Campus Virchow Clinic, D-13353 Berlin, Germany

Abstract: Therapeutical hyperthermia has been considered for cancer therapy since William Coley observed tumour remission after induction of fever by bacterial toxins at the end of the 19th century. Because fever is associated with a variety of immunological reactions, it has been suspected, that therapeutical hyperthermia might also activate the immune system in a reproducible manner and thereby positively influence the course of the disease. During the last decade, new insight has been gained regarding the immunological changes taking place during therapeutic hyperthermia. In this chapter, we review the most relevant data known about the effect of hyperthermia on the immune system with special focus on alterations induced by therapeutical whole-body hyperthermia (WBH) in cancer patients.

Keywords: whole-body hyperthermia; apoptosis; lymphocytes; cytokines; heat shock proteins; stress; review

Introduction

The medical subject headings (MeSH) database introduced the term “hyperthermia induced” in 1984 and defined it as “abnormally high temperature intentionally induced in living things regionally or whole body. It is most often induced by radiation (heat waves, infra-red), ultrasound or drugs” (http://www.ncbi.nlm.nih.gov). This definition implies that hyperthermia is a condition provoked by an external source and thus is clearly to be discriminated from other causes of elevated body temperatures, such as fever, malignant hyperthermia or other forms of heat injury like heat stroke. Regarding hyperthermia in this narrower sense, most scientific findings on the effect of external heat application on living organisms have been generated in the scope of cancer research, where preclinical and clinical approaches have been investigated for more than half a decade (reviewed in Hildebrandt et al., 2002; Wust et al., 2002; van der Zee, 2002).

Pioneering radiobiological studies established a synergism between temperature elevation and
radiation in the early 1970s (Dewey, 1994). The clinical relevance of these findings has been proven in a number of randomised clinical studies on locoregional hyperthermia, with super-agonistic effects observed for the combination of hyperthermia and certain cytotoxic drugs (Wust et al., 2002; van der Zee, 2002). In addition, a number of phase II studies suggest a beneficial effect for whole-body hyperthermia (WBH) as an adjunct to chemotherapy (Hildebrandt et al., 2005). But even if it appears to be proven that local and regional radiofrequency hyperthermia approaches are suitable to enhance the effect of radiotherapy and chemotherapy in a variety of human malignancies, most of the underlying mechanisms are still poorly understood.

Here, we summarise the most relevant data published on the effect of hyperthermia on the cellular immune system with special attention on alterations that have been observed during therapeutic WBH in cancer patients.

Basic effects of hyperthermia

Cytotoxic effects of heat

Early experiments on the cytotoxic effect of in vitro hyperthermia on cultured cells revealed a time- and dose-dependant relationship in the temperature range between 41 and 47°C. The thermal doses required to induce hyperthermic cell death varied with factor 10 between different cell types. The slope of the corresponding survival curves showed a typical shoulder, indicating a potential of cells to recover from a thermal insult (reviewed in Dewey, 1994; Dewhirst et al., 2003). Highest heat sensitivity was observed during the mitotic phase where hyperthermia induces microscopically detectable damage of the mitotic apparatus leading to inefficient mitosis and consecutive polyploidy. In contrast, G1-cells exposed to hyperthermia do not exhibit microscopically detectable changes of the mitotic apparatus (termed necrosis) but may undergo a “rapid mode of cell death” immediately after heat exposure (Vidair and Dewey, 1988). Another important finding from these early in vitro models was a systematic pattern in the temperature dependency of cell death differentiating temperatures above 43°C. This led to the concept of the “thermal isoeffect dose” (TID) by which a given thermal dose (i.e. temperature elevation for a certain time) can be converted into an “equivalent heating time at 43°C”, that has influenced hyperthermia research for the past three decades (Dewey, 1994; Dewhirst et al., 2003). Converting thermometrical data from clinical hyperthermia trials into “equivalent heating minutes at 43°C” (EM 43), significant correlations between this surrogate parameter or similar thermal parameters and clinical response parameters have been shown in various studies on local and regional hyperthermia (but not perfusional techniques or WBH) (Wust et al., 2002; Dewhirst et al., 2003). Therefore, EM 43 seems to be a valuable approach to describe the effectiveness of a clinical heat treatment.

Another groundbreaking observation from the early in vitro studies was that hyperthermia not only acts in a cytotoxic way by itself, but also sensitises tumour cells to radiotherapy and various cytostatic drugs at markedly lower temperatures than 43°C (“thermal radiosensitisation” and “thermal chemosensitisation”) (reviewed in Dewey, 1989; Hildebrandt et al., 2002; Vujaskovic and Song, 2004). The extent of thermal sensitisation in a given model can be quantified by the quotient of survival fraction of cells treated with radiation/chemotherapy alone and those treated with radiation/chemotherapy at the same dose plus heat (“thermal enhancement ratio”, TER). Both thermal radio- and chemosensitisation are reproducible in vivo and sufficiently explain that a beneficial clinical effect of radiative locoregional hyperthermia can already be observed at temperatures of 40° to 40.5°C (Myerson et al., 2004).

Although the transferability of the basic mechanisms of hyperthermic cell death on the living organism is generally assumed, the application of therapeutic hyperthermia in conjunction with radio- or chemotherapy to cancer patients is much more complex than in the pre-described preclinical models. Indeed, profound changes concerning the tumour’s blood, nutrient and oxygen supply, metabolic situation, cellular signal transduction and immunological pathways as well as pharmacological effects, have been described during or after heat treatment (reviewed in Hildebrandt et al., 2002). However, it is difficult to extract reliable
information on the effects of heat from clinical investigations with cancer patients and to separate it from other components of a multimodal treatment scheme (Hildebrandt et al., 2002).

**Molecular and cellular effects**

A large body of data concerning the cellular and molecular effects of hyperthermia exist, dating back to the 1970s. Hyperthermia has been demonstrated to affect fluidity and stability of cellular membranes and impedes the function of transmembranal transport proteins and cell surface receptors *in vitro* (Coss and Linnemans, 1996; Lepock, 2003). Therefore, it had been assumed that membrane alterations might represent an important target for the induction of heat-induced cell death. In addition, it had been proposed that hyperthermia-induced changes of cytoskeletal organisation (cell shape, mitotic apparatus, intracytoplasmatic membranes such as endoplasmatic reticulum and lysosomes) might correlate with the extent of hyperthermic cell death (reviewed in Lepock, 2003). Borrelli et al. (1986) described for the first time the appearance of “membrane blebbing” in cultured cells exposed to heat, which is a typical feature of programmed cell death (apoptosis).

In the 1960s, heat application was suggested to act similar to radiation by directly damaging nuclear DNA, thereby inducing double-strand breaks. However, it has been shown that heat does not primarily cause severe DNA damage by itself, but rather impedes the repair of radiation-induced cell damage thus boosting radiation-induced DNA fragmentation. Recent data suggest that inhibition of the “base damage repair” system may be the crucial pathogenetic step in hyperthermic radiosensitisation (reviewed in Kampinga et al., 2004).

Generally, hyperthermia exhibits its cell-killing properties by induction of apoptosis, necrosis or cell cycle arrest (reviewed in Hildebrandt et al., 2002), but detailed data on the mode of hyperthermic cell death in the living organism are not yet available. Some *in vivo* data show beneficial as well as disadvantageous effects: Regarding systemic hyperthermia application at moderately elevated temperatures, an animal study using xenotransplanted colon carcinoma revealed that long-term fever-like hyperthermia is suitable to induce significant tumour growth delay due to apoptosis. Analyses of the host tissues revealed a high rate of apoptotic cell death particularly in various lymphatic tissues, a moderately increased apoptosis within the small intestine, but not in any of the remaining organs (Sakaguchi et al., 1995; Yonezawa et al., 1996). In humans, heat-induced apoptosis is suggested to be one of the major pathogenetic mechanism mediating heat-induced developmental defects in foetuses by inducing an irreversible damage to neurogenic cells. A similar mechanism has also been proposed for heat damage of the central nervous system in adults (reviewed in Edwards et al., 2003; Sharma and Hoopes, 2003).

Taken together, data suggest that heat-induced apoptosis represents a central mechanism of heat action that may already be induced when a living organism or tumour-loaded region is subjected to mild to moderate thermal doses. But even if induction of programmed cell death in tumour tissues represents a highly desirable effect in the treatment of human malignancies, the fact that systemic hyperthermia is suitable to cause apoptosis in healthy cells such as lymphocytes raises concerns of potential adverse reactions, particularly in the scope of WBH.

**Heat shock proteins**

Hyperthermia, as well as various other stress conditions, induces the synthesis of so-called “heat shock proteins” (HSP) which may be mediated by activation of nuclear “heat shock factors” (HSF) within minutes. HSPs represent a heterogenous group of molecular chaperones consisting of at least five subgroups with different molecular mass and varying biologic function that are expressed both constitutively and stress-induced. They are usually divided into small HSPs (molecular mass <40 kDa), and the HSP 60, HSP 70, HSP 90 and HSP 100 protein families. All HSPs are able to unselectively bind to hydrophobic protein sequences liberated by denaturation, thereby preventing irreversible interaction of neighboured proteins (“chaperoning function”). In particular, the HSPs 27 and 70 are able to defend cells against
a variety of potentially lethal stimuli and thus are supposed to be “general survival proteins” (Jaattelä, 1999; Jolly and Morimoto, 2000; Kregel, 2002).

HSP synthesis, in general, increases with elevated temperatures. However, above a distinct threshold temperature (typically above 42°C), an inhibition of HSP synthesis occurs, resulting in exponential cell death. Under physiological conditions, HSPs protect cells from potentially lethal heat damage, e.g. by an increase of cellular resistance to apoptosis (Jaattelä, 1999; Jolly and Morimoto, 2000; Kregel, 2002). The major cellular pathways activated by cellular stress and important interactions with HSPs are summarised in Fig. 1. Potential cellular effects of hyperthermia are summarised in Table 1.

Effects of heat on the immune system

Recent advances have highlighted a number of mechanisms by which heat application may interact with the immune system. Some of them are suggesting a similarity between external induction of elevated temperatures and fever, a highly conserved physiological mechanism in the defence against exogenous agents and tumours. The following chapters review important aspects of the complex immunological changes induced by hyperthermia.

In vitro effects of hyperthermia on lymphocytes

A number of preclinical studies have investigated the effect of hyperthermia on human lymphocytes in vitro. The results are contradictory: Earlier investigations revealed that application of hyperthermia at different temperatures (39°C, 40°C, 42°C) impairs lymphocyte function. Especially natural-killer lymphocyte (NK-cell) activity and cytotoxic T-lymphocyte function have been found to be sensitive to heat (Azocar et al., 1982; Kalland and Dahlquist, 1983; Dinarello et al., 1986; Yang et al., 1992). Another group reported a temperature-dependent effect of hyperthermia: NK-cell activity (NKA) as well as cytotoxic activity of T-lymphocytes (T-cells) were increased at temperatures of 40°C, while incubation at temperatures of 42°C caused decreased activities of both lymphocyte subpopulations as determined in chromium release assays with appropriate target cells (Shen et al., 1994). Again, heat shock proteins seem to play an important role during these processes.

**Immunological features of heat shock proteins**

Besides their above-mentioned chaperoning function, HSPs are involved in antigen presentation, cross-presentation and tumour immunity. HSPs isolated from cancer tissues have been found to form specific complexes with tumour specific peptides that are internalised into antigen-presenting cells by specific receptors and then presented together with major histocompatibility complex (MHC) class I molecules, thereby inducing a cytotoxic T-cell-activation. It has been shown that HSPs interact with antigen-presenting cells through the CD91 receptor, inducing the re-presentation of chaperoned peptides by MHC molecules and activation of NF-kappa B (Srivastava, 2002). Multhoff and coworkers characterised solid tumour cell lines expressing a stress-inducible form of HSP 70, which mediates MHC-independent lysis. They demonstrated that a cell-surface presentation of HSP 70 may occur constitutively or heat-induced. Thereby, membrane expression of HSP 70 epitopes may represent a target for NK-cells, but also protect tumour cells from radiation damage (Multhoff, 2002; Gehrmann et al., 2005). Because of their unique immunologic features, HSPs are believed to procure more or less specific immunogenic effects induced by hyperthermia and other exogenous stimuli and have attracted particular interest for tumour vaccination strategies (Li and Dewhirst, 2002; Krause et al., 2004). The possible interactions between hyperthermia, heat shock proteins and the immune system are illustrated in Fig. 2.

**WBH-induced stress reactions**

Induction of HSPs represents only one aspect of WBH-induced stress reaction. Moreover, application of WBH is apparently associated with major physical stress, indicated by marked increases of
hearth rate, cardiac output and oxygen consumption (Faithfull et al., 1984; Kerner et al., 1999, 2003). In addition, elevated plasma levels of stress hormones have been described during WBH (Robins et al., 1987a; Kappel et al., 1991; Kearns et al., 1999) and interactions of these stress hormones with the immune system are well known (Hellstrand and Hermodsson, 1989; Madden et al., 1995; Iwakabe et al., 1998; Woiciechowsky et al., 1998; Kohm and Sanders, 2001; Elenkov and Chrousos, 2002). As physical stress (e.g. exercise) physiologically increases body temperature, it is very difficult to distinguish between hyperthermic- and stress-induced effects. Brenner et al. (1997) performed cycle ergometer exercise in a thermo neutral (23°C) or heated (40°C) climatic chamber with resting at the same temperatures as control measurement. Exercise in the thermo neutral surrounding led to significantly increased heart rates and to elevated plasma levels of the stress hormones epinephrine, nor-epinephrine and cortisol. These changes were significantly enhanced by

Fig. 1. Interactions between cellular stress pathways and heat shock proteins (adapted with permission from Repasky and Issels, 2002). Major cellular pathways activated by environmental (e.g. heat shock, hyperthermia), physiological and pathophysiological stress (e.g. fever, inflammation): (1) accumulation of misfolded proteins, (2) direct DNA damage (e.g. by UV radiation), (3) activation of p53, (4) increased levels of reactive oxygen species, (5) activation of stress activated protein kinases — SAPKs, jun n-terminal kinases — JNKs, (6) activation of Fas/Fas-L ligand. Pathways by which heat shock proteins (HSPs) interact with stress-induced alterations: (A) HSP assist proteins to acquire a native state, (B) autoregulation of HSP synthesis, (C) blockade of caspase activation by cytochrome C, (D) inhibition of caspase downstream events, (E) inhibition of SAPKs/JNKs activation, (F) inhibition of appearance of ROS species, (G) inhibition of Fas-induced apoptosis, (H) cell surface expression as antigen/antigen-presenting molecule.
additional increase of body temperatures up to 38.1°C during exercise in the heated chamber. None of the parameters changed significantly during resting in 40°C surrounding. Similar results were currently published by Rhind et al. (2004): Cycle ergometer exercise in a cold or hot water bath with rectal temperatures of 37.5 vs. 39.1°C resulted in different elevations of epinephrine and nor-epinephrine. The increase of cortisol during exercise under hot conditions was even abolished in the lower temperature group. Thus additional moderate hyperthermia enhances the secretion of stress hormones induced by physical stress.

The effect of these stress hormones on innate and acquired immunity seems to be different. There is evidence, that the innate immunity represented e.g. by monocytes and macrophages is rather stimulated by stress hormones whereas the acquired cellular immunity, represented by T-lymphocytes, may be transiently suppressed (Galon et al., 2002).

### Monocyte stimulation by WBH

The results of investigations performed during clinical trials suggest that WBH at 42°C induces the secretion of pro-inflammatory cytokines such as Interleukin-1 (IL-1), Interleukin-8 (IL-8), and tumour necrosis factor alpha (TNF-α) as well as the pro- and anti-inflammatory cytokine Interleukin-6 (IL-6) through monocytes/macrophages as part of the innate immune system (Robins et al., 1995; Haveman et al., 1996; Katschinski et al., 1999; Atanackovic et al., 2002). Similar effects have been described in patients suffering from heat stroke (Bouchama and Knochel, 2002). In addition, stimulatory effects of WBH on monocyte functions and elevated expression of monocyte markers such as CD14 and CD11b have been reported immediately after induction of moderate WBH in healthy volunteers (Zellner et al., 2002). These results are in line with the hypothesis of stress hormone-induced monocyte activation during WBH. It has been postulated that HSP may also induce secretion of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α by T-cells (Milani et al., 2002). However, it is unclear whether the results of those in vitro tests may be due to lipopolysaccarids (LPS) and LPS-associated molecules used during the tests (reviewed in Tsan and Gao, 2004).

### Transient impairment of lymphocytes during WBH

Whereas research in the context of extreme WBH at 42°C suggest an activation of innate immunity, the course of lymphocyte controlling cytokines rather indicates a switch to an anti-inflammatory pattern. Plasma levels of Interleukin-10 increased dramatically, while levels of Interleukin-12 (IL-12) and Interferon-gamma (IFN-γ) decreased during and shortly after therapy (Robins et al., 1995; Ahlers et al., 1999; Katschinski et al., 1999). Analyses of lymphocyte subpopulations revealed marked changes during therapeutic WBH: A reversible
increase of blood natural killer cells (NK-cells) as well as natural killer T-cells (NKT-cells) and γδ-T-cells — three lymphocyte subpopulations with "innate immune functions" — has been previously observed (Ahlers et al., 1998; Atanackovic et al., 2002). In addition, significant alterations of T-lymphocyte subpopulations could be found during therapeutic WBH: The number of blood CD4+ T-cells decreased, while the number of CD8+ T-cells remained unchanged, resulting in a marked drop in lymphocyte count and T4/T8 ratio. All these changes were reversible within 24 h and could not be found in a control group that received isolated chemotherapy (Ahlers et al., 1998). In addition, therapeutic WBH induced a short period of impaired T-cell proliferation as well as reduced T-cell activity — indicated by reduced serum levels of soluble interleukin-2 receptors (sIL-2R) and reduced numbers of IL-2R positive T-cells (Ahlers et al., 1998; Atanackovic et al., 2002).

Significant alterations of lymphocyte subpopulations can be observed in patients suffering from heat stroke with body core temperatures >40.1°C when compared with healthy control persons. After heat stroke, percentages of T-lymphocytes as well as the T4/T8 ratio were reduced, while percentages of natural killer (NK) lymphocytes were elevated. These changes resulted in slightly increased numbers of total lymphocytes during heatstroke (Hammami et al., 1998). Declined numbers of T-lymphocytes — without analyses of subpopulations — were also observed in cancer patients undergoing therapeutic WBH of 39°C to 40°C (Kraybill et al., 2002). Other authors examined intra-individual immunological changes in healthy volunteers whose core temperatures rose up to 39°C in a water bath. T4-cells and T4/T8 ratio decreased, while NK-cells increased reversibly in these persons. Similarly to heat stroke, these effects resulted in slightly increased numbers of total lymphocytes during moderate WBH (Downing et al., 1988; Kappel et al., 1991; Blazickova et al., 2000). The increase in NK-cell numbers came along with increased NKA during moderate WBH (Downing et al., 1988; Kappel et al., 1991). However, both studies did not calculate the NKA per NK-cell, but measured the total activity of all mononuclear cells. Therefore, the increased total NKA may just be due to the increased numbers of NK-cells (Downing et al., 1988; Kappel et al., 1991). Taken together, moderate (39°C) as well as extreme (42°C) WBH induced marked but transient shifts in lymphocyte subpopulations and -functions: NK-cells, NK-T-cells and γδ-T-cells increased significantly, while T4-cells, total T-cells and T4/T8 ratio decreased. The latter was more pronounced at 42°C, which resulted in a significant decrease of the absolute lymphocyte count at this temperature. T-cell function changed to an anti-inflammatory pattern with increase of IL-10 and decrease of IL-12 and IFN-γ, while per-cell-NKA during WBH has not been measured until now. However, IFN-γ is one of the strongest stimulators of NKA and reduced IFN-γ plasma levels may thus lead to impaired per-cell-NKA (Robertson and Ritz, 1990).

This transient anti-inflammatory phase seems to be followed by a prolonged T-cell activation: Atanackovic et al. (2002) reported increased numbers of the activation marker CD69 on T-cells and increased levels of soluble IL-2R 24 h as well as 48 h after WBH treatment.

The clinical relevance of each of these two phases of lymphocyte response to WBH has to be investigated in further studies. Currently, this phenomenon can only hypothetically be interpreted as an initial, transient compromising of the immune system with decline of T-cells and increase of anti-inflammatory cytokines as an adverse effect of hyperthermal treatment. This seems to be followed by a rather long-term activation of the acquired immune system with activation of T-cells. The latter might contribute to the beneficial effects of therapeutic hyperthermia in cancer patients.

**Stress hormones, lymphocyte shift and lymphocyte function**

The alterations observed during therapeutic WBH seem to be at least partially due to increased stress hormones accompanying the treatment procedure. Further clues on stress reactions due to this treatment come from the observation that cortisol as well as acute stress and extreme WBH are suitable to induce lymphocyte (i.e. T-lymphocyte) migration out of the blood flow towards skin, bone marrow, lung and gut. This shift might be responsible for the
pronounced decrease of T-lymphocytes due to the extreme stress during therapeutic hyperthermia (Dougherty and Frank, 1953; Amaning and Olszewski, 1994; Dhabhar et al., 1996; Pedersen and Hoffman-Goetz, 2000). Shifts from lymphocytes out of the blood flow into lymphoid tissues have also been observed in mice-experiments with long lasting moderate “fever-range” whole-body hyperthermia (FR-WBH). It is postulated, that a decrease in the number of blood lymphocytes and L-selectin-positive lymphocytes is due to an increased property of these cells to migrate into secondary lymphoid tissue, tumour tissues and sites of inflammation.

In addition, FR-WBH has been demonstrated to increase the homing potential of lymphocytes via L-selectin and α4β7 integrin (Burd et al., 1998; Evans et al., 2001; Ostberg et al., 2002; Repasky and Issels, 2002; Shah et al., 2002).

On the other hand, it has been shown that infusion of epinephrine and β2-adrenoceptor agonists may cause mobilisation of lymphocytes from endothelial cells of the blood vessels as well as from lymphatic organs into the blood flow. This effect can be abolished by simultaneous infusion of β2-antagonists (Schedlowski et al., 1996; Benschop et al., 1997; Carlson et al., 1997; Klokker et al., 1997). Density of β2-receptors is high on NK-cells, intermediate on T8-cells and low on T4-cells (Madden et al., 1995). This may lead to different catecholamine-induced mobilisation of lymphocyte subpopulations and may therefore represent one reason for the observed different courses of numbers of blood lymphocyte subpopulations during extreme hyperthermia (Ahlers et al., 1998). In addition, epinephrine decreases T4-cell proliferation (Pedersen and Hoffman-Goetz, 2000) and may thus be responsible for the observed transient impairment of T-cell proliferation described by Atanackovic et al. (2002).

The change towards an anti-inflammatory pattern of lymphocyte controlling cytokines during extreme WBH is also similar to known effects of stress hormones: Cortisol and epinephrine suppress pro-inflammatory plasma levels of IL-12 and IFN-γ as well as expression of IL-12 receptors on lymphocytes, while production of anti-inflammatory IL-10 is increased (Madden et al., 1995; Iwakabe et al., 1998; Ashwell et al., 2000; Elenkov, 2004). In addition, catecholamines and cortisol have been shown to inhibit the NKA (Madden et al., 1995; Pedersen and Hoffman-Goetz, 2000).

Taken together, activation of both the sympathetic-adrenomedullary system (SAS) and the hypothalamo-pituitary axis (HPA) as two different parts of the stress reaction, leads to marked shifts of lymphocytes between blood and other compartments. In addition, cytokine secretion, mediating lymphocyte function, changes to an anti-inflammatory pattern. Depending on the nature and duration of the stressor, these effects are mostly transient. This is in line with the above-mentioned alterations due to therapeutic hyperthermia.

**Lymphocyte apoptosis as part of immune regulation and putative effect of therapeutic hyperthermia**

Programmed cell death in lymphocytes is essential for immune regulation. Since alterations of immune responses are induced by hyperthermia, apoptosis might therefore occur after hyperthermia as well. Apoptosis represents a universal death programme that may be induced by a wide variety of stimuli in a relatively uniform way. During therapeutic hyperthermia, a variety of biochemical and immunological functions are altered. Besides direct toxic heat effects as described in the first paragraphs, many other factors could possibly induce apoptosis. The most relevant factors in the context of hyperthermia are therefore discussed in the following section.

**Stress-induced apoptosis**

As already mentioned above, systemic hyperthermia represents a condition associated with marked stress for both the single cell and the entire organism. The fact that stress might lead to immunosuppression is generally accepted. Recent investigation shows, that apoptosis of lymphocytes as well as of polymorphonuclear neutrophil leukocytes is relevantly involved in this phenomenon (Sasajima et al., 1999; Delogu et al., 2001a, b; Schroeder et al., 2001; Wang et al., 2002; Shi et al., 2003). The decrease of splenocytes
due to apoptosis in mice exposed to two 12 h periods of physical stress is as high as 30–40% (Shi et al., 2003). In lymphocytes, the mechanisms involved have been extensively investigated and seem to be critically influenced by opioids. Shi et al. (2003) demonstrated, that endogenous opioids, which are increased in stressful situations, mediate increased expression of Fas-receptor and consequently increased apoptosis of lymphocytes. On splenocytes mu-opioid-receptor-dependent increase of CD95-expression has been observed by Wang et al. (2002). This is of special interest, as opioids like fentanyl and remifentanil are used to induce analgesic sedation or general anaesthesia during therapeutical hyperthermia, and are also relevantly involved in the temperature regulation in certain circumstances associated with elevated body temperature (Romanovsky and Blatteis, 1998). In humans, the phenomenon of lymphocyte apoptosis due to stress has been reported in several different situations: After surgical trauma and general anaesthesia apoptosis of lymphocytes has been observed. Here, alterations in the mitochondrial energy metabolism seem to be involved in apoptosis induction (Delogu et al., 2001b). In patients with septic disorders, increased apoptosis of lymphocytes was reported as well (Schroeder et al., 2001). In this state of extreme stress and inflammation, certainly many factors may contribute to lymphocyte apoptosis. After major operations, T-cell apoptosis is transient: it appears 6–12 h after onset of stress and disappears 96 h after surgery (Delogu et al., 2001b; Wang et al., 2002; Shi et al., 2003).

In the course of WBH, increased lymphocyte apoptosis can be observed as well (Dieing et al., 2003). The above-described observations, together with the “stress-typical” immunological alterations suggest, that at least part of this phenomenon might be due to the stress caused by this treatment.

**Immunologically induced apoptosis**

Apoptosis of lymphocytes represents a crucial regulatory mechanism of the immune system, essential for development of immunity, maintenance of peripheral homeostasis and prevention of autoimmunity. Figure 3 gives an overview of the various instances during development; when lymphocytes may undergo programmed cell death. These processes are active in B- and T-cells. In NK-cells, the mechanisms seem to differ; therefore this phenomenon is described separately.

During development, T- and B-lymphocytes are eliminated by apoptosis when basic functions are insufficient, e.g. due to a missing antigen-receptor, or a defective IL-7-receptor with ineffective cytokine stimulation. Later in the maturation, lymphocytes are selected by proper immunologic function. An excessively avid or anergy TCR/MHC interaction leads to negative or failure of positive selection, with consecutive programmed cell death (Rathmell and Thompson, 2002). The mechanisms underlying the elimination of these cells are not entirely clear. However, the induction of nuclear steroid hormone receptor NUR77 might play a role, as it seems to regulate apoptosis through a transcriptional programme and by promoting cytochrome C release (Rathmell and Thompson, 2002). Fas and death receptor signalling through Fas-associated death domain (FADD) and caspase-8 in general are not required for thymocyte negative selection (Strasser and Bouillet, 2003). However, for the negative selection of autoreactive cells Bcl-2-related BH3 protein and receptor-induced apoptosis via TNF play a crucial role (Rathmell and Thompson, 2002; Strasser and Bouillet, 2003).

Homoeostasis of mature lymphocytes is tightly regulated by apoptosis. During early immune response, there is excessive lymphocyte expansion, which needs to be down regulated, when the antigen disappears and inflammation declines. The redundant lymphocytes are eliminated by “activation-induced cell death” (AICD) due to either cytokine withdrawal (“death-by-neglect”) or receptor-induced apoptosis (Janeway et al., 2001). After antigens and inflammatory cytokines are cleared at the end of an immune response, the missing stimulation of the activated lymphocytes leads to reduced glucose metabolism, loss of cellular ATP and mitochondrial dysfunction, and finally to programmed cell death. Death-by-neglect is caspase-independent, but regulated by proteins of the Bcl-2 family (Rathmell and Thompson, 2002).
Receptor-induced apoptosis of activated lymphocytes is mainly regulated by the TNF-receptor family. In early immune response, T-cells upregulate Fas, which acts as costimulator. With continued activation, T-cells as well express Fas-ligand (Fas-L), which leads to induction of apoptosis in the sense of suicide and/or fratricide, e.g. by cells responding to new antigenic challenge. IL-2, necessary for clonal expansion of T-cells, sensitises these cells for apoptosis in the later phase of inflammation (Krueger et al., 2003). Receptor-induced death is initiated by cleavage of the initiator caspase-8 and activation of executor caspases downstream in the cascade such as caspases-3 and -7 (Krueger et al., 2003).

During treatment of cancer patients with extreme WBH at 41.5°C to 42°C an increased rate of T-cell-apoptosis can be observed (Fig. 4). This phenomenon is transient and disappears 24 h after treatment (Dieing et al., 2003). However, activation of T-cells, measured by expression of CD69, proliferation activity and serum levels of sIL2-R occur several hours later (Atanackovic et al., 2002). During WBH, however, a short period of immunosuppression dominates (Ahlers et al., 1998; Atanackovic et al., 2002). Thus, it is unlikely, that the observed apoptosis of lymphocytes immediately after WBH is induced by activation of these cells. It might rather be an unwanted side effect, contributing to the transient impairment of the immune system.

In contrast to other T-cells, cytotoxic T-cells increase in number during therapeutical hyperthermia. Therefore cytotoxic T-cells appear to be not affected by the immunosuppressive effect of WBH. It is known, that there is some kind of AICD in these cells as well: Leite-de-Moraes and coworkers observed apoptotic cell death in activated NKT-cells that could not be inhibited by the survival factor IL-7. Fas/Fas-L interaction, as well as activation of caspases are important mechanisms in activation-induced apoptosis of NKT-cells (Leite-de-Moraes et al., 2000). However there is no data on hyperthermia-induced activation or
cell death in these cells available and the role of these cells is currently not clear.

In NK-cells, activation induced cell death differs from AICD in T-cells. In vitro, NK-cell activation by CD2 or CD16, or ligation of CD94 is followed by induction of apoptosis with detectable DNA-laddering after 2 h (Ortaldo et al., 1995; Ida and Anderson, 1998).

Cell death induced by activation in those cells is independent of TNF-α or Fas-L, as neutralising antibodies against Fas-L or TNF-α cannot inhibit apoptosis. In addition, the caspase-specific peptide inhibitor Z-VADfmk cannot prevent apoptosis, suggesting a caspase-independent pathway (Ida et al., 2003). Instead apoptotic cell death after NK-cell activation seems to involve granzyme B and nitric oxide and requires IL-2-priming (Furuke et al., 1999; Ida et al., 2003). Active granzyme B is able to cleave Bid, which in turn promotes mitochondrial dysfunction and thereby induces programmed cell death (Ida et al., 2003).

As the role of NK-cells in tumour defence is well known (reviewed in Brittenden et al., 1996), activation and possible enhancement of cytotoxicity of NK-cells by hyperthermia has been of high interest since the early 1980s. Various studies on the effect of heat on the NK-cell-function were made. These mostly in vitro experiments showed a limited potency of heat to enhance activity and cytotoxicity of lymphocytes (Azocar et al., 1982; Kalland and Dahlquist, 1983; Onsrud, 1988; Yang et al., 1992; Shen et al., 1994). A certain role of NK-cells in tumouricidal effects of fever-like hyperthermia has been shown in vivo in scid-mice bearing syngeneic breast xenographs: the number of NK-cells increased at the tumour site following 6–8 h 39.8°C hyperthermia, and tumour growth was significantly delayed. This effect could be abrogated by the anti-asialo GM1 antibody, which depletes

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Fig. 4. Apoptosis of T- and NK-cells in per cent of the referring cells before □, during ■ and 24 h after whole-body hyperthermia ▲ in the peripheral blood of patients. *Significant differences compared to the pre-therapeutic value. Notice the delayed apoptosis of NK-cells.
NK-cell activity (Burd et al., 1998). One small study showed an increase in cytotoxicity of NKT-cells up to 10% in three patients treated with WBH of 41.8°C for 1 h (Hegewisch-Becker et al., 1998). Although these data are preliminary, and the clinical consequences are unclear, the data could suggest, that the complex, potentially protective interactions of the immune system in vivo might allow NK-cell activation at temperatures as high as 41.8°C. The apoptosis rate of NK-cells in patients during therapeutic WBH is differing from the one observed in T-cells. In a study of our group, no increased apoptosis rate in NK-cells, detected by annexin-binding, could be measured during hyperthermia, but it was significantly increased when blood was taken from the same patients the following day (Fig. 4) (Dieing et al., 2003). These data are in line with in vitro experiments, where the apoptosis rate of NK-cells increased 24h after hyperthermal treatment (Dieing et al., 2000). Whether these results indicate delayed cell death after initial activation of NK-cells by the elevated temperature is questionable, since neither enhancement of NK-cell-activity during WBH nor activation-induced cell death under such conditions has been definitely shown yet.

**Drug-induced apoptosis**

In hyperthermic conditions endogenous substances as steroids or opioids are increased (Robins et al., 1987b; Romanovsky and Blatteis, 1998). When systemic hyperthermia is applied therapeutically, additional drugs such as opioids, steroids and cytostatics are regularly applied.

A series of those drugs are able to induce apoptosis in lymphocytes. One of the earliest and most appealing models to gain insight into the regulation and biochemical mechanisms of apoptosis has been the death of lymphocytes induced by glucocorticoids (GC) (Migliorati et al., 1992; Migita et al., 1997; Planey and Litwack, 2000). When systemic hyperthermia is applied therapeutically, additional drugs such as opioids, steroids and cytostatics are regularly applied. A series of those drugs are able to induce apoptosis in lymphocytes. One of the earliest and most appealing models to gain insight into the regulation and biochemical mechanisms of apoptosis has been the death of lymphocytes induced by glucocorticoids (GC) (Migliorati et al., 1992; Migita et al., 1997; Planey and Litwack, 2000). When systemic hyperthermia is applied therapeutically, additional drugs such as opioids, steroids and cytostatics are regularly applied.

For many other drugs such as cytostatics, opioids or tricyclic antidepressants, apoptosis of healthy lymphocytes is an undesired adverse effect (Xia et al., 1996; Provinciali et al., 1999; Delogu et al., 2001a; Stahnke et al., 2001).

The mechanism by which programmed cell death is induced can be different: Glucocorticoids induce loss of mitochondrial potential and subsequent activation of caspase 9. Mitogen activated protein kinases are very likely to play a role as well (Planey and Litwack, 2000; Frankfurt and Rosen, 2004). After exposure to high-dose steroids, apoptosis occurs within 2 h in lymphocytes in vivo and in vitro and the effect seems to disappear after 8 h (Migita et al., 1997).

Different mechanisms seem to be active in cytostatic drugs. A number of substances such as cisplatin, doxorubicin, mitomycin, fluorouracil and camptothecin are able to induce or increase CD95-expression and thereby the sensitivity for CD95-induced apoptosis. Doxorubicin and other antineoplastic drugs have been found to induce CD95 ligand, and the CD95 receptor/ligand interaction. Death receptor-independent cleavage of the proximal caspase-8 has also been found in drug-induced apoptosis in vitro and in vivo. However, experimental caspase-inhibition did not reduce the apoptosis rate, suggesting additional death pathways (Eichhorst et al., 2001; Stahnke et al., 2001).

Taken together, there are several factors that might explain the observed apoptosis induction in lymphocytes during WBH as stress, drugs or immunologic alterations. However, there are differences between the lymphocyte subpopulations. Of special interest is the delayed apoptosis of NK-cells, different to other lymphocyte subsets that might suggest a differentiated immunologic reaction. However, there is not enough data available to closer elucidate such a possible immunologic phenomenon.

**Conclusion**

Therapeutically utilised schemes of systemic hyperthermia are on the borderline between permanent damage and temporary affection of cellular function as well as enhancement of activation of immunologic cells. Especially NK-cells, for which a special role in tumour defence is known, might be
effector cells of a hyperthermia-enhanced immune response. On the other hand, temperatures above 41°C, often used in therapeutic settings frequently impair lymphocytes in vitro. However, a series of clinical observations demonstrate a beneficial effect of this therapeutic option for tumour patients. Currently, it remains open, whether enhanced immune response contributes to these positive effects of heat treatment under clinical conditions.

References


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