Oncothermia Journal 10:147-154 (2014)

Radiation combined with hyperthermia induces HSP70-dependent maturation of dendritic cells and release of pro-inflammatory cytokines by dendritic cells and macrophages

Petra Schildkopf[1], Benjamin Frey[1], Oliver J. Ott[1], Yvonne Ruvner[1], Gabruele Multhoff[2], Rolf Sauer[1], Rainer Fietkau[1], Udo S. Gaipl[1]

(1) Department of Radiation Oncology, Radiation Immunobiology, University Hospital Erlangen
(2) Friedrich-Alexander University of Erlangen-Nürnberg, Universitasstr. 27, 91054 Erlangen, Germany

Radiation combined with hyperthermia induces HSP70-dependent maturation of dendritic cells and release of pro-inflammatory cytokines by dendritic cells and macrophages

Abstract

Purpose
Hyperthermia (HT) treatment of cancer patients was revived over the last years and has been proven to be beneficiary for many cancer entities when applied temperature controlled in multimodal treatments. We examined whether a combination of ionizing irradiation (X-ray) and HT (41.5 C; 1h) can induce the release of heat shock protein (HSP) 70 by tumor cells and thereby lead to the activation of dendritic cells and macrophages.
Material and methods
Extracellular HSP70 was detected in supernatans (SN) of treated colorectal tumor cells by ELISA. Maturation of dendritic cells (DC) after contract with the SN was measured by flow-cytometry. Phagocytosis assays were conducted to get hints about the immune stimulating potential of the tumor cells after the respective treatments.
Results
An increased surface expression of HSP70 was observed after X-ray or X-ray plus HT while the amount of extracellular HSP70 was only increased when HT was given additionally. A high up-regulation of the co-stimulation molecule CD80 and the chemokine receptor CCR7 on DC was measured after contact with SN of X-ray plus HT treated cells. This was dependent on extracellular HSP70. Combined treatments further led to significantly increased phagocytosis rates of macrophages and DC and increased proinflammatory cytokine (IL-8 and IL-12) secretion.
Conclusion
X-ray combined with HT induces HSP70 dependent activation of immune cells and might generate a tumor microenvironment beneficial for cure.

Keywords
Radiotherapy, Hyperthermia, Heat-shock protein 70, Dendritic cells, Macrophages, Immune activation

As a single treatment, hyperthermia (HT) is not capable to replace established standard cancer treatments like radiotherapy (RT) with X-ray and treatment with chemotherapeutics. However, several experiments and studies proved that HT induces thermal chemo- and radio-sensitization. Various clinical randomized trials have already shown the effectiveness of an additional HT treatment for the disease-free survival of patients and local tumor control for various human cancer entities, without an increase in toxicity. Also in palliative settings and recurrent cancer, HT was shown to prolong local progression free survival when combined with radiochemotherapy. Most of the pre-clinical mechanistic studies on the mode of action of HT focused on the in vitro clonogenic potential of tumor cells after RT in comparison to RT plus HT. However, only few data exist dealing with the immune modulatory effect of locally applied HT when added to RT.

Heat shock proteins (HSP) are prominent proteins induced by HT treatment. Besides constitutively expressed HSP inducible forms exist, which help to protect the cell against damage after a variety of different stress stimuli like heat, oxidative stress, cytotoxic agents or radiation. Tumor cells often show up-regulated intracellular basal levels of inducible HSP. In addition, HSP70 was reported to be secreted by tumor cells and elevated sera levels were observed in cancer patients,

like HSP72 after X-ray. Extracellular HSP may act as immune activating danger signals and stimulate innate as well as adaptive immune responses. Extracellular HSP70 can bind to receptors on professional antigen presenting cells (APC) and initiate the release of pro-inflammatory cytokines and the maturation process.

Dendritic cells (DC) are potent APC. Immature DC (iDC) migrate throughout the body and take up various antigens (Ag) efficiently, but presentation of these Ag on MHC molecules is initially weak. DC must undergo a maturation process, initiated by e.g. inflammatory cytokines. Maturation is characterized by an increase in surface markers responsible for co-stimulation. The uptake of further Ag by DC is reduced during this process, which results in a decrease of the C-type lectin receptor CD209 (DC-SIGN). Furthermore, homing-receptors like CCR7 show increased expression, and different pro-inflammatory cytokines and chemokines are secreted by the mature DC. The latter migrate subsequently to draining lymph nodes, where they are able to prime antigen-specific CD8 +cytotoxic T-cells (CTL).

Tumor associated Ag can be chaperoned by HSP70, taken up and cross-presented on MHC I molecules by DC. We already demonstrated that mild HT in addition to X-ray fosters necrotic cell death and release of the danger signal HMGB1 of colorectal tumor cells. The rationale of the present study was to get preclinical immune biological mechanistic insights how temperature controlled HT (at least 41 ^{o}C for at least 1 h), when added to RT, may contribute to improved tumor control in cancer patients. In addition, the importance of the immune system in cancer therapies is long proven, but the molecular mechanisms of HT on the immune system are still elusive. Therefore, we examined how X-ray and HT influence the secretion of HSP70 by colorectal tumor cells and the subsequent activation of cells of the adaptive (DC) as well as innate (macrophages) immune system.

Materials and methods

Cell culture

Human colorectal tumor cell lines (HCT15 and SW480) and mouse colon carcinoma tumor cell line CT26.WT (CRL-2638) were used for the analyses. The cell lines were tested negative for myco-plasma with per detection kit (Minerva Biolabs, Germany).

X-ray and HT treatment of the tumor cells

Cells were irradiated with different doses (2, 5 and 10 Gy) of X-ray (120 kV, 22.7 rnA, variable time; GE Inspection Technologies, Germany). For HT, cells were treated with 41.5 ^{o}C for 1 h as described previously. The temperature variations which the tumor cells were exposed to were less than 0.2 C. Therefore, a constant and temperature stable heat delivery to the cells was assured.

Analysis of the danger signal HSP70

The quantification of HSP70 supernatantis (SN) was performed with an enzyme-linked-immunosorbent assay (ELISA) DuoSet IC Kit (R&D Systems) according to the manufacturer's instructions. The remaining cells were used for flow cytometric analysis (GalliosTM, Beckmann Coulter) of membrane-bound HSP70 with FITC-labeled anti-HSP70 cmHsp70.1 antibody (multimune, Munich, Germany).

Isolation of PBMC and generation of iDC

Human peripheral blood mononuclear cells (PBMC) were generated from heparinized whole blood samples from healthy human donors by ficoll (Biotest, Germany) density gradient separation. PBMC were sown in 6-well plates (Greiner BioOne, Germany). After 1.5 h incubation at 37 ^{o}C, non-adherent lymphocytes were removed by washing. To generate iDC, adherent monocytes were cultured for 6 days in RPMI 1640 medium (Gibco, Germany) supplemented with 10% FBS (Biochrom AG, Germany), 1% L-glutamine, 1% sodium pyruvate and 1% penicillin-streptomycin (all from Gibco, Germany) plus the appropriate differentiation cytokines: 250 U/ml IL-4 (Immuno

Tools, Germany) and 800 U/mlGM-CSF (Leukine sargramostim; Bayer Schering AG, Germany). Fresh medium was added on day 2 and 5 of culture, containing.

Flow cytometric analyses of DC activation

Activation of iDC was induced over night on day 6 with undiluted tumor cell culture SN. Afterward, DC were detached and stained for activation markers. The following fluorochrome conjugated antibodies were used: CD14-FITC and HLA-DR-Pacific blue (both Beckman Coulter), CD19-FITC, CD83-PE, and CCR7-PC7 (all from BD Pharmingen), CD80-PC7, CD86-PerCP/Cy5.5, CD25-Pacific blue, and CD40-PerCP/Cy5.5 (all from Biolegend), and DC-SIGN-APC (eBioscience). For blocking experiments, anti-HSP70 (BD Bio-sciences, USA) or anti-HMGB1 (Upstate, USA) antibody (both1:1000) was added to cell culture SN and pre-incubated for 15 min before adding the SN to the DC.

Generation of macrophages and phagocytosis assays

Human PBMC were stained red with Vibrant Dil (Invitrogen, Germany). Afterward, cells were washed and sown in 48-well plates (Greiner BioOne, Germany). After 1.5 h incubation at 37°C, nonadherent lymphocytes were removed by washing and adherent monocytes were cultured for 6 days in RPMI medium supplemented with 20 % autologous serum and 1 % penicillin-streptomycin (Gibco, Germany), to generate human monocyte-derived macrophages (MU).

On day 5 or 6, DC and MU were activated with 500 ng/ml pure LPS (Sigma, Germany), respectively, for the detection of cytokines. For phagocytosis assays, the red (Dil) stained human monocyte derived macrophages were co-incubated with the green (CFSE) stained prey. Shortly, the CFSE-labeled tumor cells were treated with RT and/or HT as described above, cultured for 24 h and then added in a five fold increased amount to the Dil stained macrophages. After 1 h of co-incubation at 37°C, the SN was removed, collected, and stored at 80^{o}C for further analysis of secreted cytokines, and the macrophages were detached from the wells for analyses by two-color flow cytometry. The double positive cells (Dil and CFSE) detected by flow cytometry are macrophages that have phagocytosed the tumor cells. To exclude a simple adhesion of the prey to the surface of the macrophages, confocal microscopic control analyses were performed. This kind of phagocytosis assay is used in many studies and was established and published by our group before. For phagocytosis of CSFE-stained and treated tumor cells by DC, the same assay was performed using Dil stained monocyte derived dendritic cells as phagocytes.

Analysis of secreted cytokines from activated macrophages and DC

SN from macrophages and DC were analyzed for cytokines by standard ELISA. The appropriate kits (TGF-b (R&D Systems), TNF-a, IL-10, IL-12p70 and IL-8 (ELISA Max Sets, all from Biolegend, San Diego, USA)) were used according to the manufacturers' instructions.

Mice experiments

The animal studies were conducted according to the principles in the guidelines for the care and use of laboratory animals and were approved by the "Regierung von Mittelfranken". For the syngeneic clonogenic in vivo experiment, female Balb/c mice (Charles River Laboratories, Sulzfeld, Germany) were injected subcutaneously with 4x10^{6} CT26 RT and/or HT treated tumor cells suspended in Ringer's solution (DeltaSelect, Germany) into the flank. Tumor outgrowth was then monitored at day 3, 10, and 14 after the injection of the tumor cells. At day 14 we stopped the analyses, since mice that have received untreated tumor cells had to be euthanized because of the large size of their tumors.

To get first in vivo hints about the immunogenicity of X-ray and/or HT-treated human HCT15 tumor cells, female Balb/c mice at an age of 4-6 weeks were immunized three times every 14 days i.p. with 4x10^{6} human viable or treated HCT15 cells. Viable human HCT15 cells, being a xenogeneic material for mice, always lead to a high immune response in mice. To compare the

effect of the treatments on the immunogenicity of the tumor cells, the increase or decrease of the specific immune response against treated HCT15 cells in comparison to viable ones was determined. For analysis of tumor cell-specific antibodies in the sera of mice, blood samples were taken before and after each immunization and analyzed by indirect immune fluorescence. Briefly, viable HCT15 target cells were incubated with the serum of the immunized mouse for 60 min at 4°C in the dark. Afterward, cells were washed and bound antibodies were analyzed by flow cytometry using a FITC-tagged F(ab')2 fragment of goat anti-mouse IgG (Invitrogen, Molecular Probes, Germany). Unspecific binding of lgG antibodies was accounted by subtraction of the values of the binding of control lgG from those obtained with serum of the immunized mice.

Statistical analyses

Statistical analyses of ELISA and flow cytometric data were performed using the unpaired Student's test. For analyses of the in vivo mouse data, the Mann-Whitney-U Test was applied in addition.

Results

X-radiation alone and in combination with HT leads to an increased exposure of HSP70 on the outer tumor cell membrane

We detected a significantly increased exposure of HSP70 on the surface of HCT15 colorectal tumor cells after X-ray or X-ray plus HT (Figure 1A). Using X-ray in combination with HT, the amount of membrane-exposed HSP70 protein was significantly decreased in comparison to irradiation only, both at an early (24 h) and late time point (not shown) after treatment. We hypothesized that the induced form of HSP70 may be released at higher rates into the extracellular space by tumor cells after an additive HT treatment.

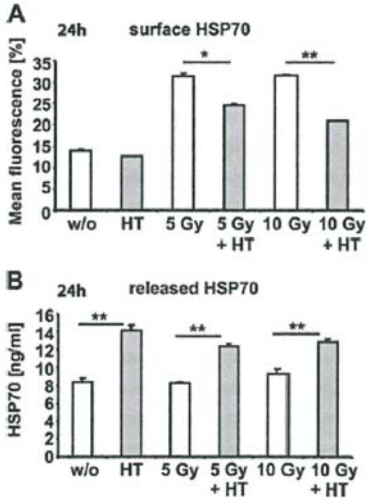

Figure 2. Membrane-exposed and extracellular HSP70 after X-ray and/or HT. Human colorectal tumor cells were treated with X-ray and/or HT. The amount of membrane-exposed HSP70 24 h after the respective treatments was detected by flow cytometry (A), and extracellular HSP70 was detected by ELISA. (B) Representative data from 1 out of 3 independent experiments, each performed in duplicates, are displayed; P < 0.05,//P < 0.01. HT: hyperthermia; w/o: mock treated control

Combinations of HT with X-radiation lead to an increased release of HSP70

We detected a significantly increased amount of extracellular HSP70 protein after HT treatment alone (Figure 1B). X-ray alone did not significantly affect the extracellular amount of HSP70. The combined treatment increased the amount of extracellular HSP70 significantly, with 2 Gy (not shown), 5 Gy, or higher (10 Gy) radiation doses (Figure 1B). At later time points, the effect of the

combinatory treatment is similar, although higher amounts of total extracellular HSP70 are achieved (not shown).

Supernatants of X-radiation plus HT treated tumor cells induce maturation of DC

Human iDC were cultured with tumor cell culture SN. The maturation marker CD83 was only slightly up-regulated on DC by all cell culture supernatants. However, the expression of CD83 was not influenced by the different cell-death initiators used (Figure 2A). Similar results were obtained for other maturation markers like CD40 (Figure 2C), HLA-DR (Figure 2D), and CD86 (Figure 2E). In contrast, CD209 was down-regulated after contact of DC with supernatants of the tumor cells (Figure 2F). The co-stimulation marker CD80 was only slightly up-regulated by the SN of X-radiation treated cells, whereas SN of HT, or most prominently SN of X-ray plus HT treated cells, significantly increased the expression of CD80 on DC (Figure 3A). The expression of the chemokine receptor CCR7 was also significantly increased after contact of DC with SN of X-ray plus HT treated cells (Figure 3B). Blocking experiments revealed that inducible HSP70 (Figure 3C), and not HMGB1 (Figure 3D), was responsible for the upregulation of CD80 as well as CCR7.

Supernatants of X-radiation plus HT treated tumor cells foster phagocytosis of tumor cells by macrophages and increase IL-8 secretion

We were interested in how X-ray and hyperthermia treatment of tumor cells influence their phagocytosis by macrophages. MU showed slightly diminished phagocytosis of tumor cells treated with X-radiation only, although the difference to untreated tumor cells was not significant. In contrast, hyperthermia treatment alone, and more pronounced in combination with X-radiation, significantly enhanced phagocytosis of tumor cells by MU (Figure 4A), which positively correlated with the radiation dose. Regarding the cytokine secretion by MU during phagocytosis, only low amounts of anti-inflammatory cytokines like IL-10 and TGF-b (not shown) were detected, while X-radiation plus HT significantly restored the secretion of the inflammatory cytokine IL-8 (Figure 4B). Another pro-inflammatory cytokine, TNF-a, was secreted in only moderate amounts and no difference between the distinct treatments was observed (not shown).

Supernatants of X-radiation plus HT treated tumor cells foster phagocytosis of tumor cells by DC and increase IL-12 secretion

DC displayed similar phagocytic activity as MU, although HT alone did not significantly increase the uptake of tumor cells by DC (Figure 4C). Combinations of X-ray with HT again increased the phagocytic activity of DC, highly significant. Activated DC secreted low amounts of IL-12p70 during phagocytosis of untreated, HT, or 2 Gy treated tumor cells (Figure 4). However, 2 Gy plus HT significantly increased the amount of secreted IL-12p70 to a similar extent as treatment with 5 Gy, 5 Gy/HT, 10 Gy, or 10 Gy/HT (Figure 4D).

Discussion

Besides targeted effects of ionizing irradiation on cellular DNA, abscopal effects arise after X-ray that may contribute to the development of anti-tumor immunity. Targeting the tumor locally by RT is capable to induce systemic effects by modulating the immune system. One main stimulus comes from the form of therapy-induced tumor cell death and the concomitant release of immune activating danger signals. In the future, immunological parameters may add value for predictive models for complete response of rectal cancer patients.

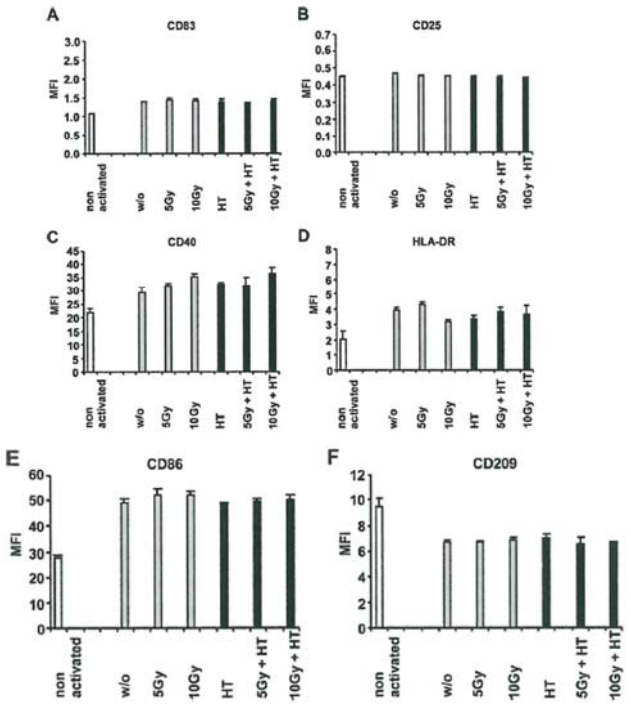

Figure 2. Maturation and co-stimulatory markers of DC after contact with SN of X-ray and/or HT treated tumor cells. Human DC were co-incubated with SN of X-ray/HT treated colorectal tumor cells. Maturation markers were detected using flow cytometry. The data are obtained from 1 out of 3 independent experiments, each performed in duplicates

Already elegantly established for imaging methods. Most of the pre-clinical assays performed focused on the in vitro clonogenic potential of tumor cells after RT in comparison to RT plus HT. We conducted in addition to the above presented results a syngeneic in vivo clonogenic assay with colorectal CT26 tumor cells in Balb/c mice. Cells treated with HT alone displayed a slightly faster tumor growth in comparison to untreated cells, indicating that HT only treatment may.

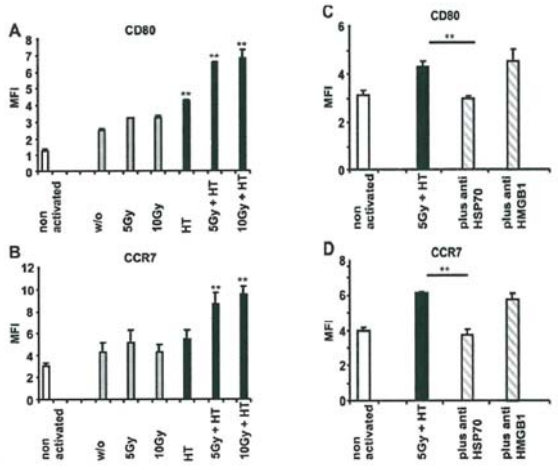

Figure 3. The role of HSP70 in up-regulation of CD80 and CCR7 on DC after contact with SN of X-ray and/or HT treated tumor cells. Human DC was co-incubated with SN of X-ray/HT treated colorectal tumor cells. CD80 (A) and CCR7 (B) expressions were detected 24 h after treatment using flow cytometry. The up-regulation of CD80 (C) and CCR7 (D) expressions was dependent on HSP70. The data are obtained from 1 out of 3 independent experiments, each performed in duplicates.//$P < 0.01$

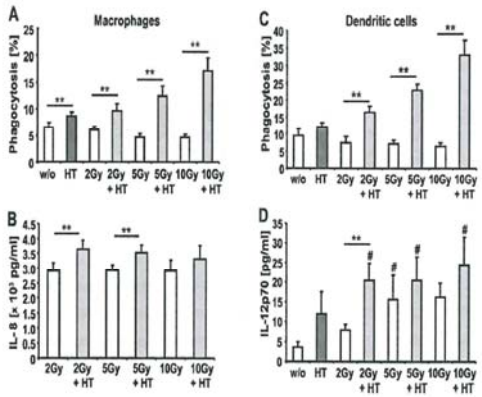

Figure 4. Phagocytosis of X-ray and/or HT treated tumor cells and cytokine secretion by macrophages and DC. Phagocytosis of colorectal tumor cells by macrophages (A) and DC (C) was measured by flow cytometry. Cytokines were detected in SN of macrophages (B) and DC (D) using ELISA technique. Representative data from one out of two independent experiments, each performed in independent pentaplicates, are displayed.//P <0.01. #P < 0.05 (against w/o)

Even foster in vivo tumor outgrowth. A significant tumor growth retardation was observed, as expected, when the tumor cells were irradiated with 5 or 10 Gy. However, a single irradiation with 2 Gy did not significantly retard the tumor outgrowth in vivo, while a combination of 2 Gy with HT was as effective as 5 Gy or 5 Gy plus HT (Supplementary Figure 1), indicating that HT added to RT has beneficial effects on tumor growth retardation. However, besides those data on clonogenic potential of tumor cells (targeted effects) only few data exist dealing with the systemic, immune sensitizing effects of HT when added to RT. HT is capable to foster programed.

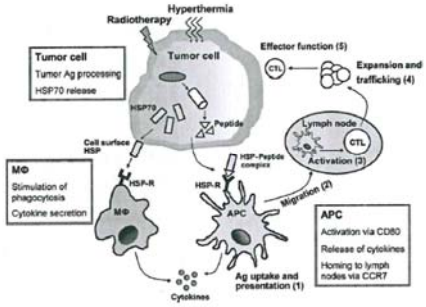

Figure 5. HT plus X-ray lead to HSP70 dependent immune activation. Inside a tumor cell, various tumor-antigens are processed into peptides, chaperoned by heat-shock protein 70 (HSP70) and secreted into the tumor microenvironment. Here, they can interact with antigen presenting cells (APC) like DC, which leads to activation, Ag uptake and presentation (1). Subsequently, after migration (2) to lymph nodes, tumor-specific CD8 + T-cells can be induced (3). After their expansion and trafficking back to the tumor (4), the T cells exert their effector function as CD8 + cytotoxic T lymphocytes (CTL; 5). In addition, HSP can directly activate macrophages, which leads to enhanced phagocytosis of tumor cells and secretion of pro-inflammatory cytokines. Modified accordingly.

Cell death pathways like necroptosis that may mediate radio-sensitizing effects. The latter were just recently discovered to be also triggered by autophagy as cell death pathway. We hypothesized in the present study that RT plus HT leads to immune activation by inducing the release of the inducible form of HSP70 from colorectal tumor cells and were further interested in its impact on DC and macrophages. We confirmed an increased surface expression of HSP70 after X-ray treatment of tumor cells. However, only HT alone or the combination of HT with X-ray led to significantly increased amounts of secreted HSP70. Secreted danger signals are mandatory as co-stimulatory molecules for the induction of an adaptive immune response. Dendritic cells present antigens (Ag) to CD4+ and CD8 + T cells. Because of its well-known function of chaperoning tumor Ag to professional APC, we analyzed the impact of an increased HSP70 release by colorectal tumor cells on the maturation of human DC. SN of X-ray and/or HT treated colorectal tumor cells led to up-regulation of several DC markers involved in maturation and co-stimulation (HLA-DR, CD40, and CD86). The co-stimulation marker CD80 and the chemokine receptor

CCR7 were only significantly enhanced on DC after contact with SN of tumor cells treated with X-ray plus HT. CD80 is the prominent co-stimulation marker of DC and up-regulation of CCR7 receptor is a prerequisite for migration of DC to lymph nodes. There, DC cross-present the chaperoned tumor Ag, activate CTL and can thereby induce specific anti-tumor immunity. We showed that DC and macrophages take up colorectal tumor cells better when they have been treated with X-ray plus HT instead of X-ray alone (Figure 4). Increased phagocytosis of tumor cells by DC subsequently can lead to increased presentation of tumor Ag.

Pro-inflammatory cytokines might result in autocrine activation of immune cells or activation of further cell types, such as natural-killer cells and T-cells. Furthermore, macrophages, like DC, are able to induce anti-tumor immunity by production of Th1 cytokines leading to subsequent stimulation of CTL. We identified that macrophages and DC secrete pro-inflammatory cytokines such as IL-8 and IL-12 after phagocytosis of X-ray plus HT treated tumor cells. The secretion of anti-inflammatory cytokines like TGF-b was low. TGF-b secretion is predominantly observed after X-radiation with low (single dose 6 0.1 Gy) and intermediate dose (single dose 61.0 Gy). It is one main player in exerting the anti-inflammatory effects of low dose X-ray. At higher single dose (> 1.0 Gy) only low amounts of TGF-b are normally observed.

Chen and colleagues recently demonstrated in an in vivo mouse model that superficial hyperthermia treatment induced the maturation of DC and suggested that the release of HSP70 is essential for this. We conclude from data presented in this manuscript that the addition of HT to the conventional treatment with X–ray retards in vivo tumor outgrowth and might increase the immune activatory potential of tumor cells, or at least maintain the one induced by X-ray, via activation of DC and stimulation of inflammatory cytokine secretion by DC and macrophages. Fig. 5 summarizes how X-ray and HT may lead to specific activation of the immune system via the induced release of HSP70.

Future research should focus on the in vivo immunogenicity of RT and/or HT treated tumor cells. We performed a first xenogeneic in vivo assay according Stach et al. testing whether treatment with RT plus HT increases the immunogenicity of human HCT15 cells in Balb/c mice. The amount of tumor-cell specific IgG antibodies did not differ significantly between untreated and HT only treated human tumor cells. Of note, cells treated with a clinically relevant single dose of 2 Gy do not show increased lgG antibody levels, but the addition of HT leads to a significant increase in tumor-specific IgG antibodies (Supplementary Figure 2). Higher dose of RT (e.g. 5 Gy) alone is capable to maintain the immunogenicity of tumor cells, clearly showing that RT treatments alone or in combination with HT do not reduce the immunogenicity of tumor cells, as e.g. described for apoptotic tumor cells that have been killed with UV irradiation. Besides exerting a general and timely restricted immune suppression, X-ray alone and in combination with HT is also capable to induce an immune activatory tumor microenvironment (Figs. 1, 3, and 4) and does not reduce the immunogenicity of tumor cells (Supplementary Figure 2).

Acknowledgments

This work was supported by the German Research Foundation (GA 1507/1-1), the German Federal Ministry of Education and Research (BMBF, m4 Cluster, 01EX1021R), and the European Commissions (NOTE-Integrated Project, TPA4 FP6, contract number 036465). We thank Dr. Niels Schaft and Dr. Jan Dörrie from the Department of Dermatology of the University Hospital Erlangen for their excellent advice about dendritic cell maturation and activation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.05.056.