Biochemical and Biophysical Research Communications 391 (2010) 1014–1020

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Application of hyperthermia in addition to ionizing irradiation fosters necrotic cell death and HMGB1 release of colorectal tumor cells

Petra Schildkopf [a], Benjamin Frey [a], Frederick Mantel [a], Oliver J. Ott [a], Eva-Maria Weiss [a], Renate Sieber [a], Christina Janko [b], Rolf Sauer [a], Rainer Fietkau [a], Udo S. Gaipl [a,*]

[a] Department of Radiation Oncology, University Hospital Erlangen, Friedrich-Alexander University of Erlangen-Nürnberg, Germany
[b] Department for Internal Medicine 3, Institute for Clinical Immunology, Friedrich-Alexander University of Erlangen-Nürnberg, Germany

ARTICLE INFO

Article history:
Received 26 November 2009
Available online 5 December 2009

Keywords:
Hyperthermia
Ionizing irradiation
Colorectal cancer
Necrosis
HMGB1
Anti-tumor immunity

ABSTRACT

Colorectal cancer is the second leading cause of death in developed countries. Tumor therapies should on the one hand aim to stop the proliferation of tumor cells and to kill them, and on the other hand stimulate a specific immune response against residual cancer cells. Dying cells are modulators of the immune system contributing to anti-inflammatory or pro-inflammatory responses, depending on the respective cell death form. The positive therapeutic effects of temperature-controlled hyperthermia (HT), when combined with ionizing irradiation (X-ray), were the origin to examine whether combinations of X-ray with HT can induce immune activating tumor cell death forms, also characterized by the release of the danger signal HMGB1. Human colorectal tumor cells with differing radiosensitivities were treated with combinations of HT (41.5 °C for 1 h) and X-ray (5 or 10 Gy). Necrotic cell death was prominent after X-ray and could be further increased by HT. Apoptosis remained quite low in HCT 15 and SW480 cells. X-ray and combinations with HT arrested the tumor cells in the radiosensitive G2 cell cycle phase. The amount of released HMGB1 protein was significantly enhanced after combinatorial treatments in comparison to single ones. We conclude that combining X-ray with HT may induce anti-tumor immunity as a result of the predominant induction of inflammatory necrotic tumor cells and the release of HMGB1.

Introduction

Colorectal cancer is the second leading cause of death in developed countries, with an incidence of 50,000 new cases per year in Germany. In early stages, radical surgery is the most important curative treatment. Late stages require (neo-) adjuvant therapies. Randomized clinical studies already proved positive effects of combined treatments with radiotherapy (RT) and hyperthermia (HT) for colorectal cancers [1]. Furthermore, no negative effects of HT on the quality of life was observed, when HT was combined with RT and chemotherapy (CT) [2]. The biological rationales of HT comprise direct cytotoxicity, systemic effects, chemosensitization, radiosensitization, and immune modulation. Many of the biological data available for HT are dealing with the proliferative behavior of tumor cells. SW480 colorectal tumor cells display low and HCT 15 cells intermediate radiosensitivity, as determined by the clonogenic assay [3]. This assay defines the "surviving fraction" of cells treated with different radiation doses. However, it cannot give information about the viability of the irradiated tumor cells or the occurring type of cell death [4].

By now, little is known about the forms of tumor cell death and the release of danger signals after combined treatment with ionizing irradiation (X-ray) and HT. First experiments performed by our group have given a hint that the amount of intracellular HMGB1 decreases in colorectal HCT 15 tumor cells after treatment with X-ray plus HT [5]. It is important to distinguish between pre-apoptotic, apoptotic, and necrotic forms of cell death [6,7]. Besides the massive disturbance of plasma membrane morphology, the plasma membrane of apoptotic cells is known to remain ion selective for a certain time. Apoptotic cells are known to exert non-inflammatory or even anti-inflammatory effects. Necrosis, in contrast, is defined by a loss of membrane integrity of the cells, leading to inflammation: various released cellular components act as damage-associated-molecular-patterns (DAMP) or danger signals [8]. The latter foster the maturation and migration of DC and may lead to specific T cell activation [9,10]. However, also very early or pre-apoptotic

* Corresponding author. Address: Department of Radiation Oncology, Radiation Immunobiology, University Hospital Erlangen, Friedrich-Alexander University of Erlangen-Nürnberg, Universitätsstr. 27, 91054 Erlangen, Germany. Fax: +49 9131 85 39335.
E-mail addresses: petra.schildkopf@uk-erlangen.de (P. Schildkopf), benjamin.frey@uk-erlangen.de (B. Frey), frederick.mantel@web.de (F. Mantel), oliver.ott@uk-erlangen.de (O.J. Ott), eva-maria.weiss@uk-erlangen.de (E.-M. Weiss), renate.sieber@uk-erlangen.de (R. Sieber), christina.janko@uk-erlangen.de (C. Janko), rolf.sauer@uk-erlangen.de (R. Sauer), rainer.fietkau@uk-erlangen.de (R. Fietkau), udo.gaipl@uk-erlangen.de (U.S. Gaipl).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.12.008

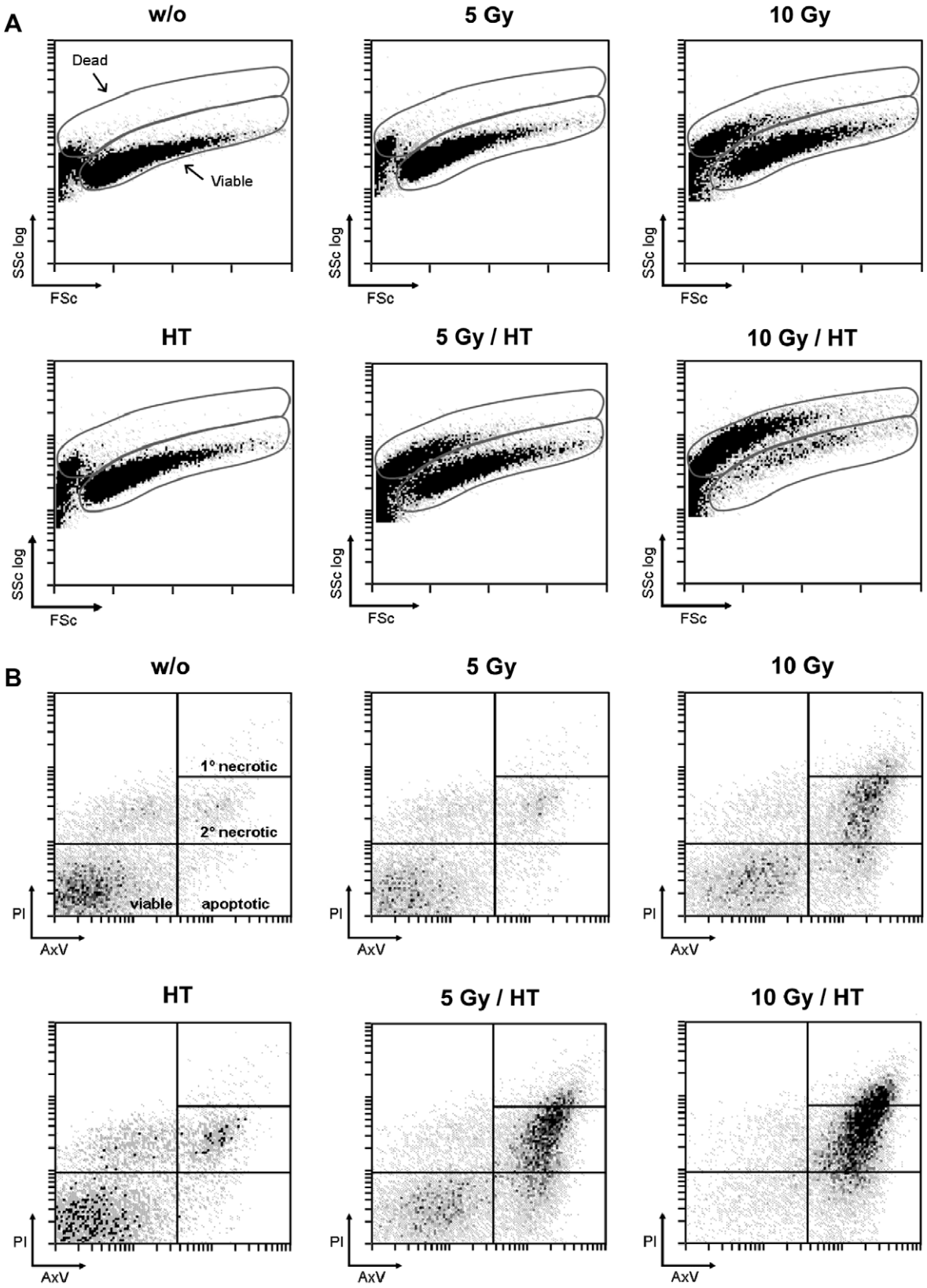

Fig. 1. Morphological changes and AxV/PI binding properties of colorectal tumor cells after treatment with ionizing irradiation and/or hyperthermia. HCT 15 colorectal tumor cells were treated with ionizing irradiation (5 or 10 Gy), HT (41.5 °C for 1 h), or a combination of both. Seventy-two hours after treatment, the cells were stained with AxV-FITC/PI, and cell death was analyzed by flow cytometry. Viable and dead cells were defined by their FSc/SSc (A) as well as by their AxV-FITC/PI binding properties (B). One representative set of experiments out of four is displayed. 1°, primary; 2°, secondary, AxV, annexinV; Gy, Gray; HT, hyperthermia; PI, propidium iodide; w/o, mock treated control.

cells can lead to immune activation by exposing eat-me signals like calreticulin for DC [11].

Hyperthermia leads to the induction of heat-shock proteins (HSP). Inhibiting the expression of intracellular HSP may sensitize tumor cells to HT [12]. The expression level of HSP can determine the fate of cells in response to death stimuli [13]. When released, HSP may induce cross-presentation of tumor antigens by DC and lead to activation of natural killer cells [14]. HSP may therefore be considered as danger signals, according to the theory that the immune system is more concerned with entities that cause damage than with those that are foreign [15]. The most prominent danger signal is the chromatin associated high-mobility group box1 (HMGB1) protein which is constitutively expressed in all cells. It leaks from necrotic cells to alert the body that damage has occurred. Necrotic cells deficient for HMGB1 were shown to have a strongly reduced ability to activate antigen-presenting cells [16].

In the current study we focused on the effect of a half-weekly dose (5 Gy) and a cumulative weekly dose (10 Gy) in tumor therapy of ionizing irradiation applied alone or together with HT (41.5 °C for 1 h) on the induction of colorectal tumor cell death, on tumor cell cycle arrest, as well as on the release of HMGB1 by SW480 and HCT 15 colorectal tumor cells. Our experiments aim to establish insights how HT and X-ray may lead to the development of long-lasting anti-tumor immunity as already shown for certain chemotherapeutics like oxaliplatin [17].

Materials and methods

Cell culture. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% foetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 1% sodium pyruvate, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in 5% CO_2 and 90% humidity. The human colorectal adenocarcinoma cell line SW480 was obtained from the American Type Culture Collection (ATCC; Wesel, Germany), and the human colorectal adenocarcinoma

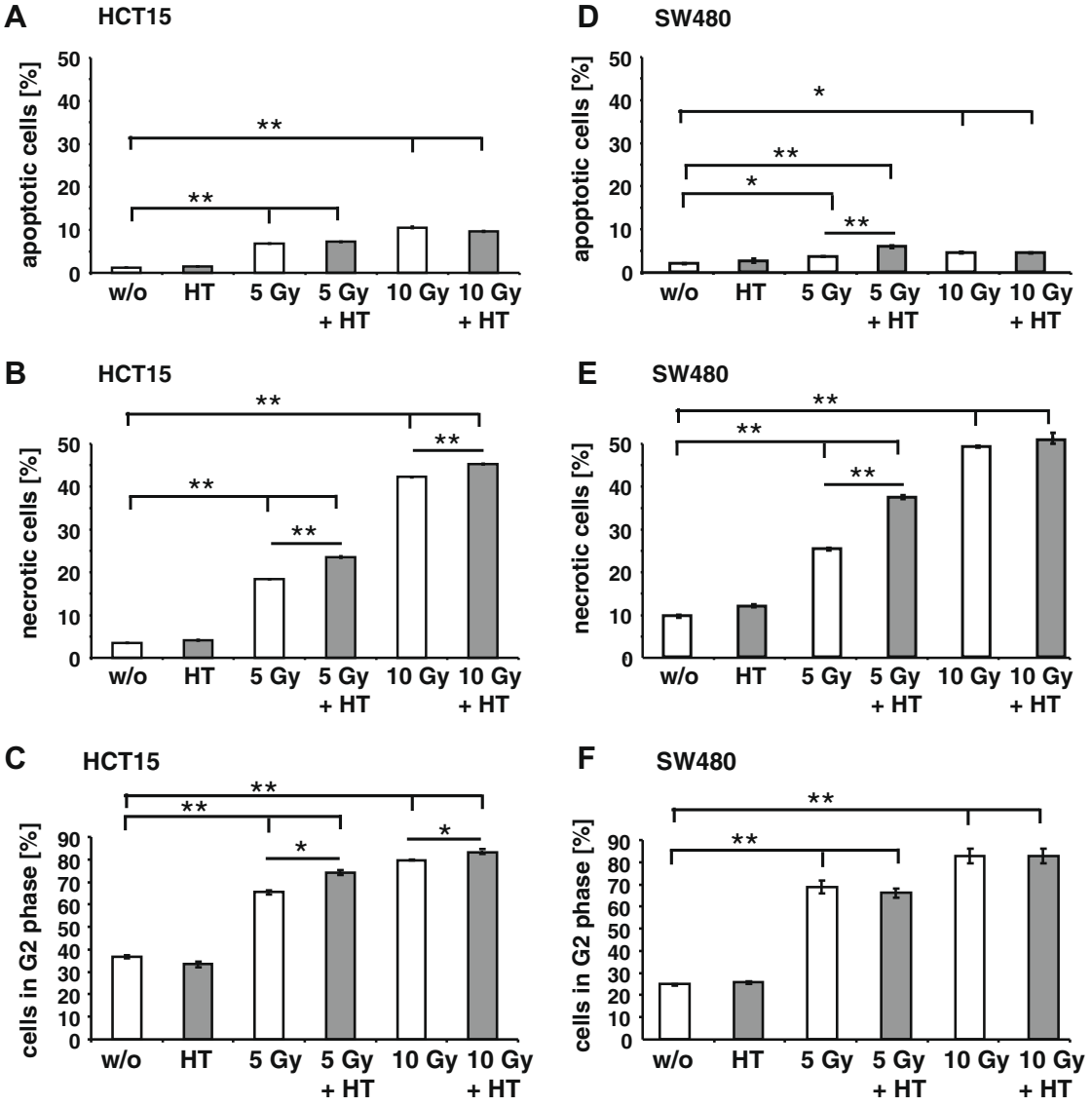

Fig. 2. Apoptosis, necrosis, and cell cycle stop of colorectal tumor cells treated with ionizing irradiation and/or hyperthermia. HCT 15 (A–C) or SW480 (D–F) colorectal tumor cells were treated with ionizing irradiation (5 or 10 Gy), HT (41.5 °C for 1 h), or a combination of both. Seventy-two hours after irradiation, the cells were stained with AxV-FITC/PI, and cell death was analyzed by flow cytometry. The percentage of apoptotic and necrotic (primary plus secondary necrotic) tumor cells after treatment is displayed in (A, D) and (B, E), respectively. The tumor cells were further stained 24 h after treatment with PI in presence of the detergent Triton and the cell cycle was analyzed by flow cytometry. The percent of HCT 15 cells (C) and SW480 cells (F) being in the G2-phase of the cell cycle is displayed. The data are obtained from 4 independent experiments, each performed in duplicates. $^{*}P < 0.05$, $^{**}P < 0.01$. HT, hyperthermia; Gy, Gray; w/o, mock treated control.

cell line HCT 15 from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany).

Induction and detection of cell death. Cells were irradiated with 5 or 10 Gy of X-ray (120 kV, 22.7 mA, variable time; GE Inspection Technologies, Hürth, Germany). For hyperthermia, cells were treated in a home made HT chamber placed in a cell incubator. The variations of the temperature to which the cells were exposed were less than 0.2 °C. The cells remained at 41.5 °C for 1 h. For combined applications, cells were stored at 37 °C for 4 h between X-ray and HT treatment, as it is common in clinical application.

Flow cytometry was used to detect death of colorectal tumor cells after X-ray and/or HT treatment. During cell death, characteristic changes of the morphology can be detected by forward scatter (FSc)/side scatter (SSc) properties of the cells [18]. To distinguish apoptotic from primary and secondary necrotic cells, the exposure of phosphatidylserine (PS) by apoptotic and necrotic cells was analyzed by binding of FITC-labeled AnnexinV (AxV), and necrosis was differed from apoptosis by co-staining with propidiumiodide (PI) as described previously [19]. PI is able to penetrate into cells which have lost their membrane integrity and intercalates DNA. Primary necrosis was differed from secondary necrosis by the intensity of the PI staining [18]. The cell cycle was analyzed by staining with PI in the presence of the detergent Triton X-100 [20]. Analyses by flow cytometry were performed with an EPICS XL MCL (Coulter, Fullerton, USA) apparatus.

Analysis of the extracellular amount of the danger signal HMGB1. The presence and amount of HMGB1 was analyzed with an anti-HMGB1 antibody (dilution 1:2000; Upstate, New York, USA) by Western blot using standard protocols. To detect extracellular HMGB1, cell culture medium was changed to medium with 0.1% β-mercaptoethanol (50 mM) and without FBS before treatment with X-ray and HT, since HMGB1 was shown to be sensitive to oxidation [21] and to form complexes with various proteins [22]. After one day of culture, the cell culture supernatants were concentrated using Amicon Ultra 15 and Amicon Microcon YW-100 (Millipore, Schwalbach/TS, Germany) centrifugal filter devices. Afterwards, equal amounts of each filtrate were used for Western blot analysis.

Statistical analyses. Data are obtained from four independent experiments, each performed in duplicates. Statistical analyses were performed using the Student's *t*-test. A *P*-value of <0.05 was considered as significant (∗) and one of <0.01 as highly significant (∗∗).

Results

Necrosis is the prominent form of cell death after treatment of colorectal tumor cells with X-ray alone or in combination with hyperthermia

Primary data of the FSc/SSc properties of HCT 15 cells 72 h after treatment with X-ray, HT, or a combination of both are displayed in Fig. 1A. Two distinct cell populations can be defined: viable and dead cells. During cell death, the morphology of dying cells changes, resulting in decreased FSc and increased SSC properties. Combinations of RT and HT significantly increased the dead cell population in comparison to single treatments (Fig. 1A).

Representative dot plots of the AxV/PI binding properties of HCT 15 tumor cells 72 h after treatment with X-ray and/or HT are shown in Fig. 1B. Living cells are negative for AxV and for PI binding. Apoptotic cells expose PS on their cell surface and are therefore positive for AxV binding, but stain negative for the DNA intercalating PI. Primary necrotic cells with full DNA content are positive for AxV binding and show high PI fluorescence. Late apoptotic/secondary necrotic cells have degraded DNA that was in part already secreted with apoptotic blebs. Therefore, those cells show intermediate PI fluorescence and are positive for AxV binding. Treatment with HT alone slightly increased the amount of secondary necrotic cells compared to the mock treated control. Combinations of X-ray with HT led to an increase of mainly secondary necrotic cells in early stages of the apoptotic program.

Hyperthermia enhances the amount of necrotic colorectal tumor cells induced by X-ray

We performed four independent experiments each in duplicates and determined viable (AxV neg./PI neg.), apoptotic (AxV pos./Pi neg.), and necrotic (AxV pos./PI intermediate and high pos.) HCT 15 or SW480 cells after single and combinatory treatments. As shown in Fig. 2, X-ray alone led to a highly significant increase of necrotic HCT 15 (Fig. 2B) and necrotic SW480 (Fig. 2E) cells in comparison to mock treated cells or cells treated with HT only. The percentage of necrotic cells was also highly significantly increased when colorectal tumor cells were treated with a combination of 5 Gy and HT in comparison to X-ray alone. The same effect was to be observed in HCT 15 cells applying 10 Gy, however, to a lesser extent. The amount of apoptotic cells was significantly increased after X-ray or after combination of X-ray plus HT (Fig. 2A and D). However, the amount of apoptotic cells was generally low 24 h (not shown), 48 h (not shown), and 72 h (Fig. 2) after the treatment in comparison to that of necrotic cells. Notably, the less radiosen-

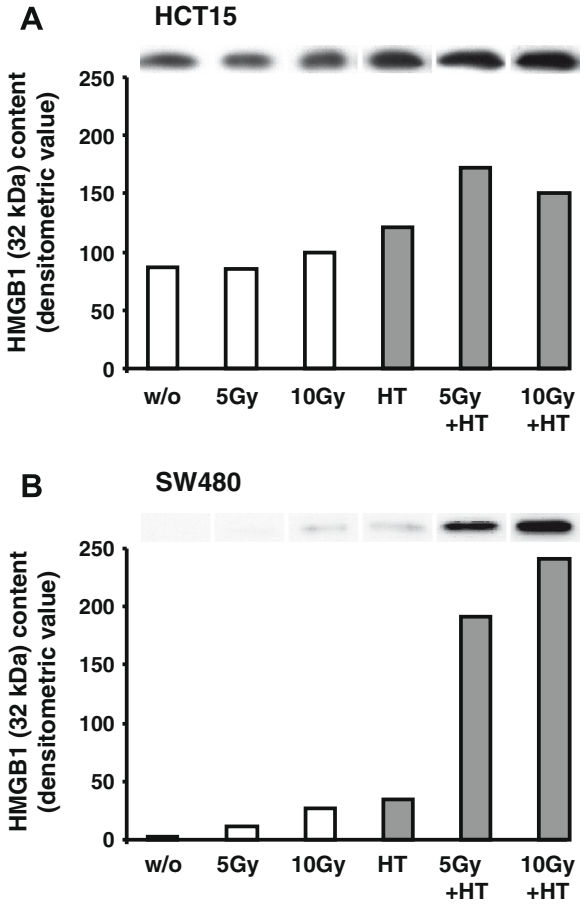

Fig. 3. Release of the danger signal HMGB1 by colorectal tumor cells after treatment with ionizing irradiation and/or hyperthermia. HCT 15 (A) or SW480 (B) colorectal tumor cells were treated with ionizing irradiation (5 or 10 Gy), HT (41.5 °C for 1 h), or a combination of both. Twenty-four hours after irradiation, the amount of extracellular HMGB1 was analyzed with anti-HMGB1 antibody in concentrated cell culture supernatants using standard Western blot protocols. The figure shows a representative blot from three independent experiments. HT, hyperthermia; Gy, Gray; w/o, mock treated control.

sitive colorectal SW480 tumor cells and the intermediate radiosensitive HCT 15 colorectal tumor cells behaved similar in regards to cell death induction.

X-ray and combinations with hyperthermia arrest colorectal tumor cells in the radiosensitive G2 cell cycle phase

Ionizing radiation is known to block cells in the G2-phase of the cell cycle, in which cells are highly sensitive to further, fractionated irradiation. Treatment of HCT 15 cells with 5 or 10 Gy led to a significantly increased cell cycle arrest in the G2-phase after 24 h, compared to the mock treated control cells (Fig. 2C). HT alone did not influence the cell cycle. However, combinations of HT with irradiation, 5 or 10 Gy, led to a slight, but significant increase of cells in the G2-phase. This was not observed in SW480 cells where combinations of HT with irradiation led to a similar G2 cell cycle stop compared to irradiation only (Fig. 2F).

The amount of extracellular HMGB1 is increased after combinatory treatment of colorectal tumor cells with X-ray and hyperthermia

The amount of HMGB1 in the extracellular space was determined with Western blot analysis of concentrated supernatants (see Materials and methods) of colorectal tumor cells one day after treatment with X-ray and/or HT. Combining irradiation with hyperthermia led to a significant increase in the amount of HMGB1 in the supernatants of HCT 15 (Fig. 3A) and SW480 cells (Fig. 3B). Hyperthermia treatment after irradiation led to a higher increase of HMGB1 compared to applications of HT before RT (not shown). In HCT 15 cells, the basal level of HMGB1 in the supernatant of mock treated cells was higher in comparison to SW480 cells.

Discussion

The immune system plays an important role in cell homeostasis: dead, abnormal, or precancerous cells are recognized and removed by phagocytes. Malignant tumor cells evade this immune surveillance through immunoselection and immunosubversion [23]. Combinatory tumor therapies should aim to stimulate a specific immune response against residual cancer cells. One possibility to achieve this is based on the induction of immunogenic tumor cell death forms. Even very early apoptotic cells can become immunogenic when they expose eat-me signals for DC, like it was demonstrated for calreticulin [6]. However, apoptotic cells are often immune suppressive while necrotic cells stimulate the immune system [7]. Necrotic tumor cells release various danger signals (e.g. HMGB1, HSP, monosodium urate, ATP) which act as inflammatory substances in the tumor microenvironment [8]. Released ATP by tumor cells was just recently shown to be a very potent activator of the NLRP3 inflammasome in DC, finally leading to adaptive immunity against tumors [24]. During apoptosis, damage-associated-molecular-patterns (DAMP) stay hidden in the cells because of an intact cellular membrane. We have shown that HT in combination with X-ray leads to mainly necrotic cell death forms of colorectal tumor cells. The latter release HMGB1, being a well known DAMP. On the one hand, DAMP can be recognized by cells

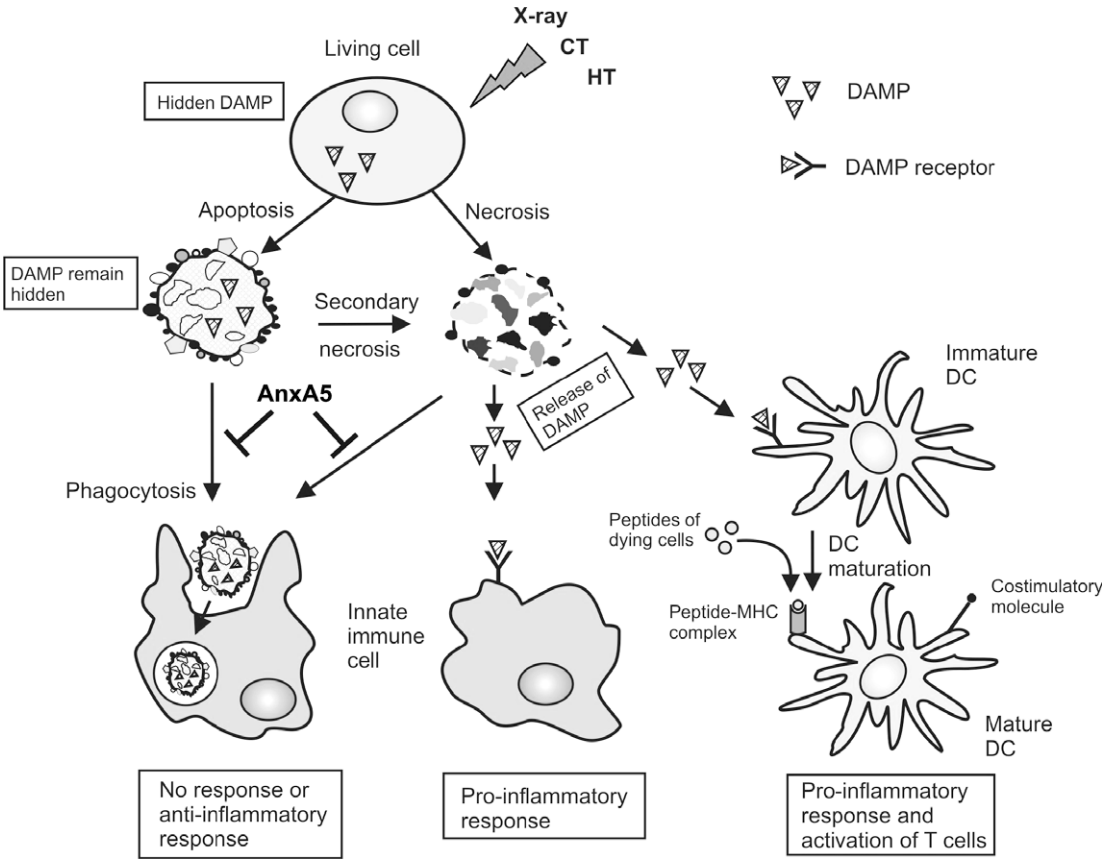

Fig. 4. Immune activation by necrotic tumor cells. A scheme how therapy (RT with X-ray, CT, and HT)-induced necrotic tumor cells can contribute to the development of anti-tumor immunity is displayed (modified from [33,34]). In brief, DAMP are released by necrotic cells and lead to immune activation, either through the innate immune system (middle) or the adaptive immune system (right). Apoptotic cells do not release DAMP and are therefore mostly non- or even anti-inflammatory (left). Proteins like AnxA5, which are capable to disturb the PS-dependent clearance by macrophages of dying tumor cells, could further improve the anti-tumor immune response. AnxA5, annexinV; CT, chemotherapeutics; DAMP, damage-associated-molecular-pattern; DC, dendritic cell; HT, hyperthermia; RT, radiotherapy; X-ray, ionizing irradiation.

of the innate immune system. On the other hand, DAMP can interact with receptors like the receptor for advanced glycation end products on immature DC. HMGB1 then mediates cross-presentation of antigenic peptides of dying tumor cells by matured DC. Together with co-stimulatory molecules, T- and B-cells are activated starting a specific anti-tumor immune response (Fig. 4).

We have to understand how classical cancer therapies (CT, RT, and HT) modulate the immune system. New technologies like magnetic resonance (MR)-guided hyperthermia assure that temperatures above 41 °C are achieved inside the tumor tissue. Hyperthermia has been shown feasible and well tolerated in rectal cancers, either in combination with CT followed by RT [25], or in combination with RT in patients with primarily locally advanced tumors [1,26] as well as in pre-irradiated patients with local recurrence [27]. Milani and colleagues showed that pre-irradiated patients with recurrent rectal cancer can be treated with RCT in combination with regional HT, showing high efficacy and acceptable toxicity [28].

In the current study we demonstrated that the prominent form of cell death of colorectal tumor cells after combinatory treatment with RT and HT is necrosis, while the rates of apoptosis remain quite low. We conclude that after the treatments, dying tumor cells go rather fast into secondary necrosis instead of preceding their apoptotic program. Furthermore, they may conduct a form of programmed necrosis, the so called necroptosis [29], since the expression of RIP1 kinase was upregulated after treatment with RT plus HT (own unpublished data). In human colorectal tumors HMGB1 often is over expressed in the cytoplasm. The release of HMGB1 can on the one hand foster tumor progression [30], but on the other hand also stimulate an anti-tumor immune response; even in brain tumors [31]. In the current work we showed for the first time that the amount of extracellular HMGB1 is increased when treating colorectal tumor cells with combinations of HT and RT.

Cell lines with different radiosensitivities, reflecting the high variability of one tumor entity, might vary in cell death induction. As shown here, cells with low (SW480) and intermediate (HCT 15) radiosensitivity are comparable in their forms of cell death after RT and/or HT treatment. Our experiments further revealed that combinations of HT with RT lead to a cell cycle arrest of the tumor cells in the radiosensitive G2-phase. Using a dose of 20 Gy of X-ray, the G2 arrest was even prolonged for 24 h in colorectal tumor cells treated additionally with HT (not shown). A precise understanding of the impact of cell-cycle progression on drug or irradiation-induced cell death might help to enhance the efficacy of chemotherapeutics. Cisplatin led to a higher number of dead tumor cells when applied in the G2 cell cycle phase in comparison to the G1 phase [32].

Taken together, therapy (X-ray plus HT)-induced necrotic cell death of and HMGB1 release by colorectal tumor cells can contribute to the development of specific and long-lasting anti-tumor immunity. Therefore, hyperthermia should be taken into account in multimodal anti-cancer therapies in addition to RT and CT.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the ELAN Fond [ST-08.06.30.1] of the Friedrich-Alexander University of Erlangen-Nuremberg and by the German Research Foundation [DFG HE 4490/3-1 and SFB 643]. We thank Dr. Sennewald and Dipl.-Ing. Martin Wadepohl for the fruitful discussions about the technique and application of temperature-controlled hyperthermia.

References

[1] B.A. Berdov, G.Z. Menteshashvili, Thermoradiotherapy of patients with locally advanced carcinoma of the rectum, Int. J. Hyperthermia 6 (1990) 881–890.
[2] T. Schulze, P. Wust, J. Gellermann, B. Hildebrandt, H. Riess, R. Felix, B. Rau, Influence of neoadjuvant radiochemotherapy combined with hyperthermia on the quality of life in rectum cancer patients, Int. J. Hyperthermia 22 (2006) 301–318.
[3] C. Rodel, J. Haas, A. Groth, G.G. Grabenbauer, R. Sauer, F. Rodel, Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor, Int. J. Radiat. Oncol. Biol. Phys. 55 (2003) 1341–1347.
[4] G. Kroemer, L. Galluzzi, P. Vandenabeele, J. Abrams, E.S. Alnemri, E.H. Baehrecke, M.V. Blagosklonny, W.S. El-Deiry, P. Golstein, D.R. Green, M. Hengartner, R.A. Knight, S. Kumar, S.A. Lipton, W. Malorni, G. Nunez, M.E. Peter, J. Tschopp, J. Yuan, M. Piacentini, B. Zhivotovsky, G. Melino, Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009, Cell Death Differ. 16 (2009) 3–11.
[5] P. Schildkopf, R. Holmer, R. Sieber, O.J. Ott, C. Janko, F. Mantel, B. Frey, R. Fietkau, U.S. Gaipl, Hyperthermia in combination with X-irradiation induces inflammatory forms of cell death, Autoimmunity 42 (2009) 311–313.
[6] M. Obeid, A. Tesniere, F. Ghiringhelli, G.M. Fimia, L. Apetoh, J.L. Perfettini, M. Castedo, G. Mignot, T. Panaretakis, N. Casares, D. Metivier, N. Larochette, P. Van Endert, F. Ciccosanti, M. Piacentini, L. Zitvogel, G. Kroemer, Calreticulin exposure dictates the immunogenicity of cancer cell death, Nat. Med. 13 (2007) 54–61.
[7] R.E. Voll, M. Herrmann, E.A. Roth, C. Stach, J.R. Kalden, I. Girkontaite, Immunosuppressive effects of apoptotic cells, Nature 390 (1997) 350–351.
[8] M.E. Bianchi, DAMPs, PAMPs and alarmins: all we need to know about danger, J. Leukoc. Biol. 81 (2007) 1–5.
[9] D. Messmer, H. Yang, G. Telusma, F. Knoll, J. Li, B. Messmer, K.J. Tracey, N. Chiorazzi, High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization, J. Immunol. 173 (2004) 307–313.
[10] I.E. Dumitriu, M.E. Bianchi, M. Bacci, A.A. Manfredi, P. Rovere-Querini, The secretion of HMGB1 is required for the migration of maturing dendritic cells, J. Leukoc. Biol. 81 (2007) 84–91.
[11] D.R. Green, T. Ferguson, L. Zitvogel, G. Kroemer, Immunogenic and tolerogenic cell death, Nat. Rev. Immunol. 9 (2009) 353–363.
[12] J.H. Wang, M.Z. Yao, J.F. Gu, L.Y. Sun, Y.F. Shen, X.Y. Liu, Blocking HSF1 by dominant-negative mutant to sensitize tumor cells to hyperthermia, Biochem. Biophys. Res. Commun. 290 (2002) 1454–1461.
[13] C. Garrido, S. Gurbuxani, L. Ravagnan, G. Kroemer, Heat shock proteins: endogenous modulators of apoptotic cell death, Biochem. Biophys. Res. Commun. 286 (2001) 433–442.
[14] G. Multhoff, Activation of natural killer cells by heat shock protein 70, Int. J. Hyperthermia 25 (2009) 169–175.
[15] P. Matzinger, The danger model: a renewed sense of self, Science 296 (2002) 301–305.
[16] P. Rovere-Querini, A. Capobianco, P. Scaffidi, B. Valentinis, F. Catalanotti, M. Giazzon, I.E. Dumitriu, S. Muller, M. Iannacone, C. Traversari, M.E. Bianchi, A.A. Manfredi, HMGB1 is an endogenous immune adjuvant released by necrotic cells, EMBO Rep. 5 (2004) 825–830.
[17] A. Tesniere, F. Schlemmer, V. Boige, O. Kepp, I. Martins, F. Ghiringhelli, L. Aymeric, M. Michaud, L. Apetoh, L. Barault, J. Mendiboure, J.P. Pignon, V. Jooste, P. van Endert, M. Ducreux, L. Zitvogel, F. Piard, G. Kroemer, Immunogenic death of colon cancer cells treated with oxaliplatin, Oncogene (2009).
[18] U.S. Gaipl, S. Kuenkele, R.E. Voll, T.D. Beyer, W. Kolowos, P. Heyder, J.R. Kalden, M. Herrmann, Complement binding is an early feature of necrotic and a rather late event during apoptotic cell death, Cell Death Differ. 8 (2001) 327–334.
[19] G. Koopman, C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals, M.H. van Oers, Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis, Blood 84 (1994) 1415–1420.
[20] I. Nicoletti, G. Migliorati, M.C. Pagliacci, F. Grignani, C. Riccardi, A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry, J. Immunol. Methods 139 (1991) 271–279.
[21] H. Kazama, J.E. Ricci, J.M. Herndon, G. Hoppe, D.R. Green, T.A. Ferguson, Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein, Immunity 29 (2008) 21–32.
[22] V. Urbonaviciute, B.G. Furnrohr, C. Weber, M. Haslbeck, S. Wilhelm, M. Herrmann, R.E. Voll, Factors masking HMGB1 in human serum and plasma, J. Leukoc. Biol. 81 (2007) 67–74.
[23] L. Zitvogel, A. Tesniere, G. Kroemer, Cancer despite immunosurveillance. immunoselection and immunosubversion, Nat. Rev. Immunol. 6 (2006) 715–727.
[24] F. Ghiringhelli, L. Apetoh, A. Tesniere, L. Aymeric, Y. Ma, C. Ortiz, K. Vermaelen, T. Panaretakis, G. Mignot, E. Ullrich, J.L. Perfettini, F. Schlemmer, E. Tasdemir, M. Uhl, P. Genin, A. Civas, B. Ryffel, J. Kanellopoulos, J. Tschopp, F. Andre, R. Lidereau, N.M. McLaughlin, N.M. Haynes, M.J. Smyth, G. Kroemer, L. Zitvogel, Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors, Nat. Med. 15 (2009) 1170–1178.
[25] B. Hildebrandt, P. Wust, J. Drager, L. Ludemann, G. Sreenivasa, S.G. Tullius, H. Amthauer, P. Neuhaus, R. Felix, H. Riess, Regional pelvic hyperthermia as an

adjunct to chemotherapy (oxaliplatin, folinic acid, 5-fluorouracil) in pre-irradiated patients with locally recurrent rectal cancer: a pilot study, Int. J. Hyperthermia 20 (2004) 359–369.
[26] B. Rau, P. Wust, J. Gellermann, W. Tilly, M. Hunerbein, J. Loffel, H. Stahl, H. Riess, V. Budach, R. Felix, P. Schlag, Phase II study on preoperative radio-chemo-thermotherapy in locally advanced rectal carcinoma, Strahlenther. Onkol. 174 (1998) 556–565.
[27] J.H. Juffermans, P.E. Hanssens, W.L. van Putten, G.C. van Rhoon, J. van Der Zee, Reirradiation and hyperthermia in rectal carcinoma: a retrospective study on palliative effect, Cancer 98 (2003) 1759–1766.
[28] V. Milani, M. Pazos, R.D. Issels, V. Buecklein, S. Rahman, K. Tschoep, P. Schaffer, R. Wilkowski, E. Duehmke, M. Schaffer, Radiochemotherapy in combination with regional hyperthermia in preirradiated patients with recurrent rectal cancer, Strahlenther. Onkol. 184 (2008) 163–168.
[29] J. Hitomi, D.E. Christofferson, A. Ng, J. Yao, A. Degterev, R.J. Xavier, J. Yuan, Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway, Cell 135 (2008) 1311–1323.
[30] J.E. Ellerman, C.K. Brown, M. de Vera, H.J. Zeh, T. Billiar, A. Rubartelli, M.T. Lotze, Masquerader: high mobility group box-1 and cancer, Clin. Cancer Res. 13 (2007) 2836–2848.
[31] J.F. Curtin, N. Liu, M. Candolfi, W. Xiong, H. Assi, K. Yagiz, M.R. Edwards, K.S. Michelsen, K.M. Kroeger, C. Liu, A.K. Muhammad, M.C. Clark, M. Arditi, B. Comin-Anduix, A. Ribas, P.R. Lowenstein, M.G. Castro, HMGB1 mediates endogenous TLR2 activation and brain tumor regression, PLoS Med. 6 (2009) e10.
[32] S. Mueller, M. Schittenhelm, F. Honecker, E. Malenke, K. Lauber, S. Wesselborg, J.T. Hartmann, C. Bokemeyer, F. Mayer, Cell-cycle progression and response of germ cell tumors to cisplatin in vitro, Int. J. Oncol. 29 (2006) 471–479.
[33] H. Kono, K.L. Rock, How dying cells alert the immune system to danger, Nat. Rev. Immunol. 8 (2008) 279–289.
[34] L.E. Munoz, B. Frey, F. Pausch, W. Baum, R.B. Mueller, B. Brachvogel, E. Poschl, F. Rodel, K. von der Mark, M. Herrmann, U.S. Gaipl, The role of annexin A5 in the modulation of the immune response against dying and dead cells, Curr. Med. Chem. 14 (2007) 271–277.