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γ-Ray irradiation induces B7.1 costimulatory molecule neoexpression in various murine tumor cells

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Abstract The use of gene-modified tumor cells as a strategy for active immunotherapy is currently undergoing intensive fundamental and clinical research. Most clinical trials use γ-ray-irradiated tumor cells as vaccine, although little is known about the effects of irradiation on the immunogenicity of tumor cells. In particular, no data have been reported so far concerning the effects of γ-ray irradiation on the expression of B7 molecules in tumor cells. In this paper, we show a neoexpression of the B7.1 molecule after γ-ray irradiation in tumor cell lines from different tissues, while the B7.2 molecule remains unexpressed in all the cell lines tested. Furthermore, the induction of B7.1 molecule membrane expression after irradiation is shown to result from the neoexpression of B7.1 mRNA, and to be reproduced with H_2O_2 oxidative stress. These data could explain the enhanced immunogenicity of many tumor cells after irradiation, and could lead to new immunotherapy protocols.

Key words Costimulatory molecule, B7.1 · Irradiation · Immunology · Tumor cells

Introduction

Tumor cells engineered to express various immunomodulatory molecules have shown a promising potential as vaccines in various experimental animal models [32]. The results of these studies have provided the groundwork for numerous clinical trials of gene therapy in cancer patients [1, 32]. Notably, most protocols in humans are performed with irradiated tumor cells for safety reasons. It is known that irradiation of tumor cells often increases their inherent immunogenicity [13], but the mechanisms of this effect have not been extensively studied. Some authors have reported that MHC class I or II molecules show enhanced expression upon irradiation of tumor cells [21, 25], while others report an up-regulation of interleukin(IL)-8 or the costimulatory molecule intercellular adhesion molecule (ICAM) 1 [36, 37].

However, no data showing the effects of γ-ray irradiation on the expression of B7.1 (CD80) and B7.2 (CD86) molecules in tumor cells have been reported to date. B7.1 and B7.2 molecules are essential membrane proteins giving a costimulatory signal necessary to activate T cells [17, 19]. Recognition of MHC class I peptide presentation without B7 costimulation leads to T cell anergy [18]. B7.1 and B7.2 are normally expressed in activated "professional" antigen-presenting cells (APC) [15, 20]. However, B7.1 expression has also been reported in various tumor cell lines [4] and in melanoma cells of some patients [22]. The expression of B7.1 in tumor cells may have physiological significance since many authors have reported that gene transfer of B7.1 or B7.2 molecules into different tumor cell lines enables them to generate a strong immune response [9, 23, 34]. In view of these data, we decided to study the effect of γ-ray irradiation on B7.1 and B7.2 membrane expression in different tumor cell lines.

We demonstrate that γ-ray irradiation selectively induces B7.1 expression in several tumor cell lines, while B7.2 remains unexpressed after irradiation in all cell lines tested. These data may have important implications for immunotherapy protocols.

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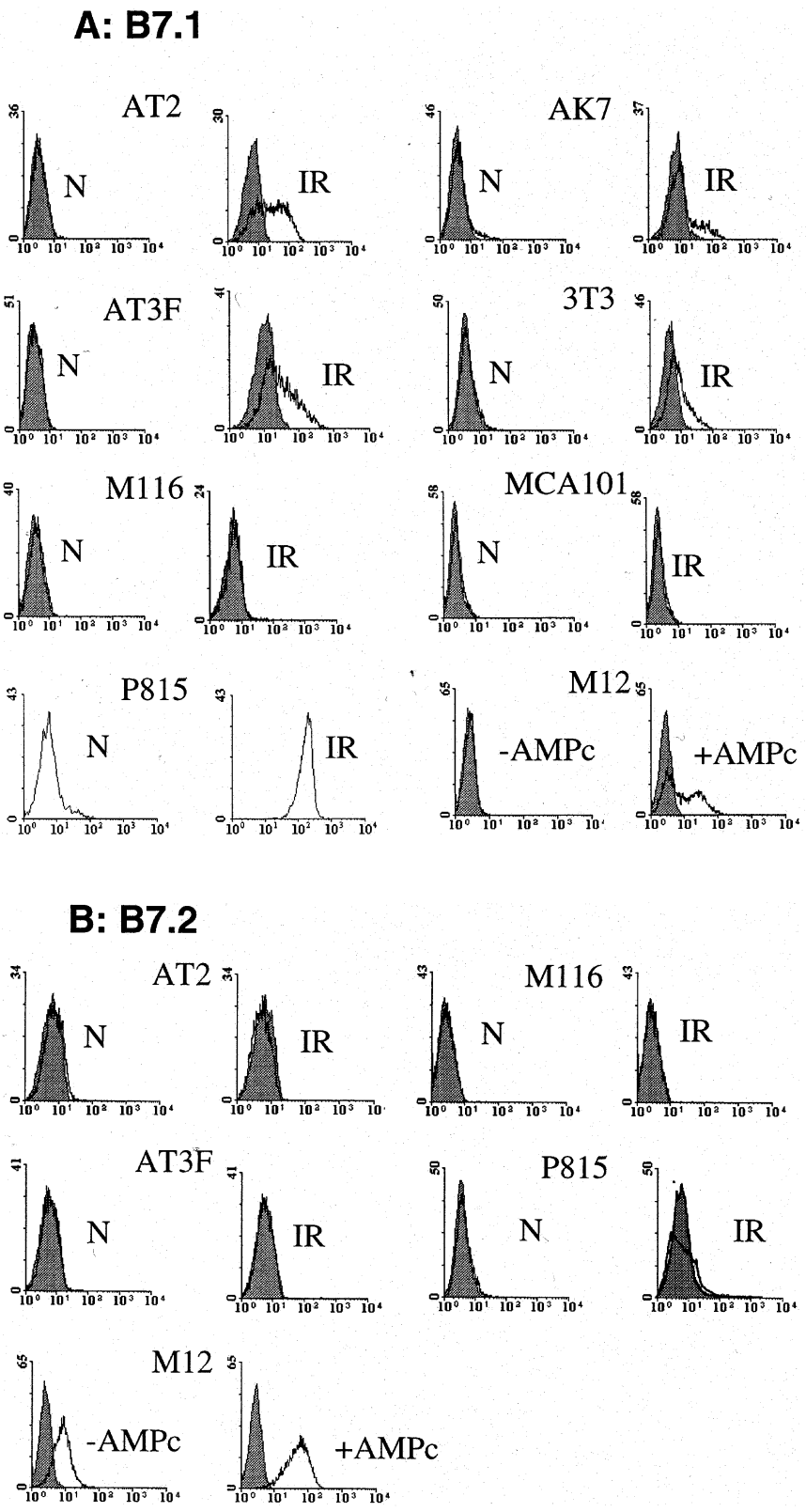

Fig. 1A, B Effect of γ-ray irradiation on the expression of B7.1 (**A**) and B7.2 (**B**) molecules in tumor cell lines. Fluorescence-activated cell sorting (FACS) analyses were performed under normal culture conditions (*N*) or 3 days after γ-ray irradiation (*IR*) on different tumor cells. M12B lymphoma cells were used as controls, stimulated or not with 300 μg/ml dibutyryl-cAMP for 48 h. Cells were immunostained with either rat anti-(mouse CD80) monoclonal antibody (*white*) or isotype control (*black*), followed by phycoerythrin-labeled goat anti-(rat IgG). This procedure resulted in a very low non-specific staining for P815 cells (not shown), requiring the use of 2.4G2 antibody, which blocks non-antigen-specific binding to the mouse FcγII/III receptors. It was followed by the staining with the rat anti-(mouse CD80) monoclonal antibody directly coupled to fluorescein (Figs. 1, and 2). Experiments were performed three times for each cell type and gave similar results

Materials and methods

Cell lines and culture

Hepatoma cell lines mhAT3F and mhAT2 were derived from transgenic mice synthesizing the simian virus 40 (SV40) large T and small t antigens in the liver under the control of antithrombin III gene regulatory sequences [3, 26]. The leiomyoma cell line mM116 was derived from transgenic mice synthesizing the SV40 large T and small t antigens in the uterus under the control of a short region of calbindin D-9K gene regulatory sequences [6]. Hepatoma cells were grown in Ham F12/Dulbecco's modified Eagle's medium (DMEM)/glutamax (Gibco, Chagrin Falls, Ohio, USA) medium supplemented with 1% penicillin/streptomycin, 0.1 mM insulin, 1 mM dexamethasone, 1 mM triiodothyronine and 5% (v/v) fetal calf serum. Leiomyoma cells were grown in M199 glutamax (Gibco) medium supplemented with 1% penicillin/streptomycin, 0.5 mM insulin, 1 mM transferrin, 1 mM selenium, 0.1 μM estradiol and 10% (v/v) fetal calf serum. MCA-101 fibrosarcoma [29], P815 mastocytoma and M12 lymphoma [35] cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 4 mM glutamine, 1 mM sodium pyruvate and I % combined antibiotics. AK7 mesothelioma cells were grown in DMEM (Gibco) supplemented with 10% fetal calf serum and 1% combined antibiotics. NIH3T3 cells were grown in the same medium supplemented with 10% newborn calf serum and 1% combined antibiotics.

Stress induction

For heat-shock treatment, cells were exposed to 42 °C for 2 h and then returned to 37 °C, and further incubated for 7 h (total incubation time 9 h) (for RNA extraction) or 18 h (for immunostaining with anti-CD80). For oxidative stress, H_2O_2 was added to the medium at 1–2 mM final concentration and cells were further incubated at 37 °C for 9 h until RNA extraction or 18 h until immunostaining. Irradiation with 45 Gy by a ^{137}Cs source was performed 2 days before RNA extraction or 3 days before immunostaining, except for the study of B7.1 time-dependent regulation in irradiated mhAT3F cells (Fig. 2). The different incubation times were consistent with the known time-dependent effects of each stimulus on other genes. As a control, M12 B lymphoma cells were stimulated with 300 μg/ml dibutyryl-cAMP for 48 h [35].

Reverse transcription/polymerase chain reaction (RT-PCR)

Total RNA were isolated from cell lines by lysis in 7 M guanidine hydrochloride, followed by phenol extraction [10] and reverse transcription into cDNA as described by Akli et al. [2]. The PCR buffer contained 500 mM of each dNTP, 2 units Taq polymerase (Gibco BRL) and 200 ng each primer. PCR primer sequences used were as follows: B7.1 = 5′-CAGTTGATGCAGGATACACC-3′ and 5′-TGTTCTTGCTATCAGGAGGG-3′; β-actin = 5′-CGTGGGCCGCCCTGGCACCA-3′ and 5′-TGTGCCTTAGGGTTCAGGGGGG-3′. Amplification products were run on a 1% (w/v) agarose gel.

Immunostaining

Cells were suspended in phosphate-buffered saline/2 mM EDTA, washed twice and incubated with either rat anti-(mouse CD80) monoclonal antibody (Pharmigen, San Diego, USA) or the isotype control (rat IgG2a purified protein; Caltag, San Francisco, USA), followed by phycoerythrin-labeled goat anti-(rat IgG) (Immunotech, Marseille, France). Immunostained cells were analyzed on a FACScan flow cytometer (Coulter, Miami, USA) or visualized by fluorescence microscopy. Staining of P815 cells required the preliminary use of 2.4G2 antibody (Pharmingen) which blocks non-antigen-specific binding to the mouse FcγII/III receptors. It was followed by staining with the rat anti-(mouse CD80) monoclonal antibody directly coupled to fluorescein (Pharmingen).

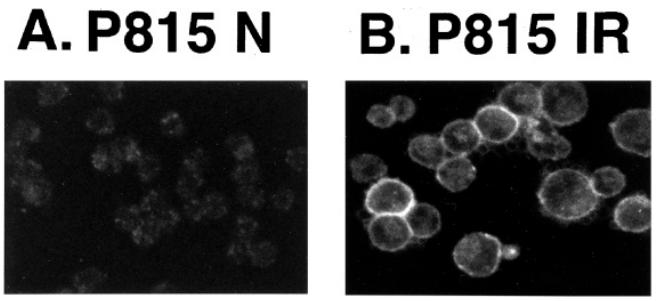

Fig. 2A, B B7.1 immunostaining in live or irradiated P815 mastocytoma cells. *N* normal conditions, *IR* irradiated cells

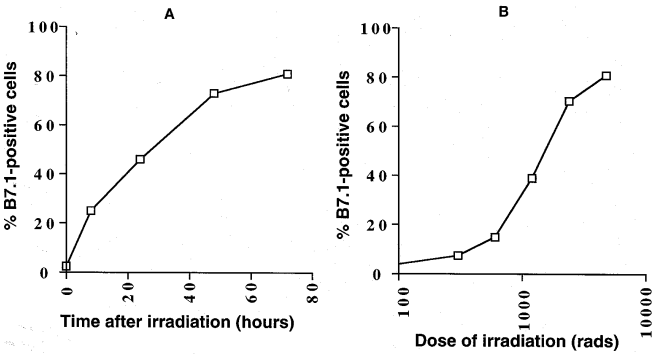

Fig. 3A, B B7.1 time- and dose-dependent regulation in irradiated mhAT3F hepatoma cells. FACS analyses were performed 8–72 h after irradiation with 48 Gy (**A**) or 72 h after mhAT3F cells were irradiated with 30–48 Gy (**B**). Data shown are from one of two independent experiments giving similar results

Results

Irradiation-induced B7.1 expression in tumor cell lines

Mouse cell lines originating from different tissues and with different differentiation levels were selected to test the effects of γ-ray irradiation on B7.1 membrane expression; these include hepatoma cell lines mhAT3F and mhAT2 [3, 26], the leiomyoma cell line mM116 [6], fibrosarcoma cell line MCA-101 [29], mesothelioma cell line AK7 (submitted), mastocytoma cell line P815 and fibroblast cell line NIH3T3. B7.1 expression was tested by FACS analysis 3 days after irradiation with 45 Gy (Fig. 1A). None of the tumor cell lines tested expressed B7.1 constitutively on the membrane. However, irradiation induced the neoexpression of the B7.1 molecule in P815 cells (95% cells staining positive), in mhAT2 and mhAT3F hepatoma cells (60% positive), and, to a lesser extent, in NIH3T3 fibroblast cells (30% positive) and AK7 mesothelioma cells (25% positive). mM116 leiomyoma cells and MCA-101 fibrosarcoma cells failed to express B7.1 on the membrane after irradiation. M12 lymphoma cells were used as positive controls for B7 membrane expression; they expressed B7.1 on the membrane when treated with dibutyryl-cAMP

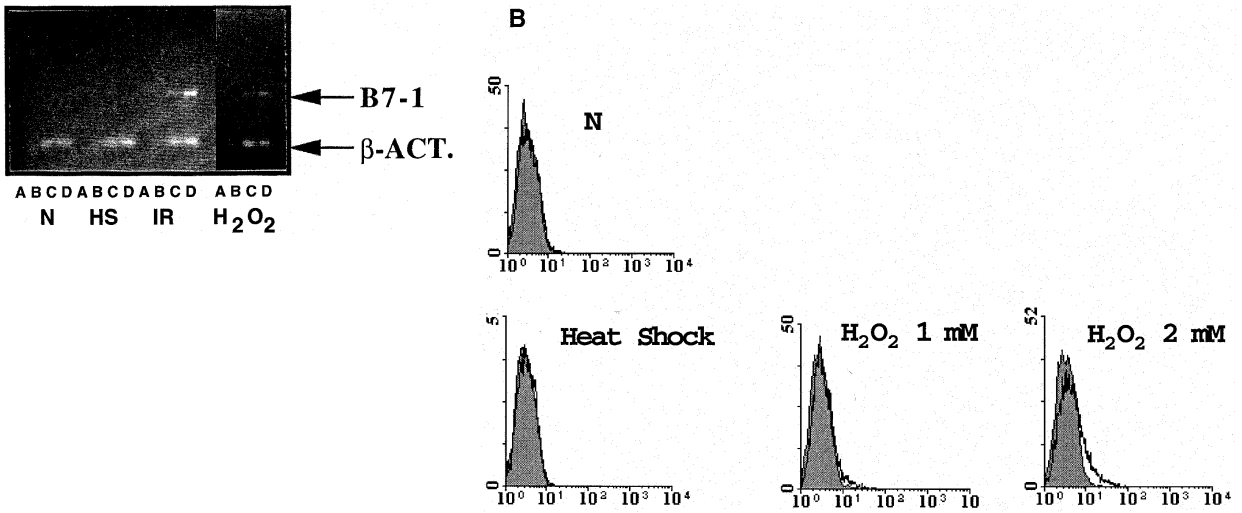

Fig. 4A, B B7.1 expression in mhAT3F hepatoma cells submitted to different stress conditions. **A** Reverse transcriptase/polymerase chain reaction (20–35 cycles) was performed on RNA extracts from mhAT3F cells under normal conditions (*N*) or submitted to heat shock (*HS*), irradiation (*IR*), or 1 mM H_2O_2. β-Actin oligonucleotides were used in separate reactions for control of amplification. **B** FACS analyses of B7.1 membrane expression in mhAT3F cells under normal conditions (*N*) or submitted to heat shock (*HS*), irradiation (*IR*), or in the presence of 1–2 mM H_2O_2. Cells were immunostained with either rat anti-(mouse CD80) monoclonal antibody (*white*) or isotype control (*black*), followed by phycoerythrin-labeled goat anti-(rat IgG). Data shown are from one of three independent experiments giving similar results

for 48 h before cell staining (Fig. 1A), in agreement with previously published data [35].

In contrast, none of the tumor cells tested (mhAT2, rnhAT3F, P815 or mM116) were B7.2-positive, either before or after irradiation (Fig. 1B). B7.2 was expressed in unstimulated M12 lymphoma cells and this basal expression was further enhanced after treatment with dibutyryl-cAMP. Watts et al. have reported a slight membrane expression of B7 molecules in some unstimulated cells derived from M12, when the CTLA4Ig fusion protein that recognizes both B7.1 and B7.2 was used [35]. The immunostaining of B7 in unstimulated M12 cells may be due to B7.2 expression. Membrane expression of B7.1 protein was further established by fluorescence microscopy, as shown for irradiated P815 cells (Fig. 2b).

Analysis of B7.1 neoexpression in mhAT3F hepatoma cells

mhAT3F hepatoma cells were selected for the further study of B7.1 neoexpression after irradiation. B7.1 time- and dose-dependent expression was studied by fluorescence-activated cell sorting analysis (Fig. 3). Surface expression of B7.1 protein was seen 8 h after irradiation, and gradually increased for 3 days (Fig. 3A). B7.1 neoexpression was shown to be induced by 3 Gy irradiation and to be dose-dependent (Fig. 3B).

Reactive oxygen species are important mediators of the effects of γ-ray irradiation on gene activation [24]. Furthermore, activation of transcriptional factors by reactive oxygen species resembles that induced by heat shock [31]. Both of these conditions are known to act via different though overlapping pathways and to induce heat-shock proteins [24, 30, 31]. We thus compared the effects of radiation on B7.1 expression to those of hydrogen peroxide (H_2O_2) and heat shock (Fig. 4). RT-PCR revealed no B7.1 mRNA in non-stressed mhAT3F cells, nor in mhAT3F cells submitted to 42 °C heat shock for 2 h (Fig. 4A). In contrast, both irradiation and H_2O_2 stress induced B7.1 mRNA and protein expression (Fig. 4A,B). In all cases, a qualitative correlation was found between B7.1 mRNA and protein expression, suggesting that B7.1 expression is primarily regulated at the RNA level, at least under the conditions used in these experiments.

Discussion

Irradiation induces B7.1 neoexpression in tumor cells

B7.1 membrane neoexpression was induced by γ-ray irradiation in several tumor cell lines, but not all. This is the first report to our knowledge that γ-ray irradiation induces B7.1 membrane expression in tumor cells. Irradiation selectively induced B7.1 neoexpression, since B7.2 membrane expression was not detected in any of the cell lines tested. B7.1 and B7.2 costimulatory molecules, the respective functions of which have not been conclusively discriminated [28, 33], are known to be differentially regulated in APC upon stimulation [20]. The inducibility of B7.1 protein expression by irradiation could depend, to some extent, on the tissue of origin of each tumor cell line. Notably, B7.1 expression was detected in tumor cell lines of fibroblast (NIH3T3) and hepatic (mhAT2 and mhAT3F) origin, two tissue types known to have antigen-presentation capabilities. Consistent with the idea that these two cell types can act as APC in some circumstances, B7.1 protein expression has been reported in specific liver tissues of patients with chronic hepatitis C [27], and the embryo-derived fibroblast cell line C3H10T1/2 has been shown to

express B7.1 constitutively [4]. However, the fibrosarcoma cell line MCA-101, like the leiomyoma cell line M116, failed to express B7.1 after irradiation.

B7.1 membrane expression in tumor cells has been observed in some other rare circumstances. Melanoma cells in some patients present a slight B7.1 membrane protein expression that can be increased by various cytokines [22]. Also, a murine B16 melanoma cell line engineered to express the MHC class II molecule has been reported to show neoexpression of B7.1 and B7.2 proteins during *in vivo* tumor rejection [5]. Antonia et at. [4] found B7.1 protein expression in a pancreatic cell line derived from transgenic mice expressing the SV40 T antigen. The authors suggested that oncogenic transformation could induce B7.1 expression, but that this expression was unable to result in an effective antitumor immune response because the associated immunotolerance to transgenic cells. However in our study, mhAT2, mhAT3F and mM116 cell lines, derived from tumors of transgenic mice, did not constitutively express B7.1 protein on the membrane (Fig. 1).

Possible mechanisms supportive of irradiation-induced B7.1 neoexpression

It appears from our data and other reports in the literature that B7.1 expression occurs in a number of situations, including transformation and immune rejection (*in vivo*), and γ-ray irradiation (*in vitro*), all of which have in common an increased generation of reactive oxygen species. In our experiments, we were able to reproduce the effects of γ-ray irradiation using H_2O_2. Thus, it is possible that the neoexpression of B7.1 mRNA we report is induced by the activation of reactive-oxygen-species-responsive transcriptional factors, like early growth response factor 1 (egr-1) or NF-κB-like factors, known to be involved in the up-regulation of other genes upon irradiation and H_2O_2 stress [7, 11]. The promoter region and one cell-type-specific enhancer of the B7.1 gene have recently been characterized [14, 38]; of interest, while no egr-1 elements were found, one essential region of the enhancer bound several members of the NF-KB family in B7.1-positive cells [14, 38]. These proteins could be involved in the neoexpression of B7.1 in irradiated tumor cells. However, incubation of mhAT3F cells with the anti-oxidant *N*-acetyl-L-cysteine (10 μM) prior to irradiation did not prevent B7.1 neoexpression (data not shown), so the involvement of reactive oxygen species in the effects of irradiation is still unclear. In addition, UV-B irradiation, known to induce reactive oxygen species, has been shown to reduce the up-regulation of B7.1 in activated monocyte APC [16], which contrasts with our observation.

Another possibility could be the involvement of heat-shock factor, known to induce up-regulation of heat-shock proteins under stress [31]. However, mhAT3F cells submitted to heat shock did not express B7.1 mRNA or protein (Fig. 4), while expressing high levels of hsp72 proteins (data not shown). In addition, no heat-shock elements have been found in the B7.1 promoter or enhancer regions [14, 38]. These data suggest that heat-shock factor is not involved in the irradiation-induced expression of B7.1.

B7.1 mRNA induction

B7.1 mRNA and protein were both induced by irradiation and H_2O_2, albeit not by heat shock (Fig. 4). This qualitative correlation between B7.1 mRNA and protein expression suggests that B7.1 expression was primarily regulated at the mRNA level under these conditions, as has been demonstrated for activated professional APC [15]. In contrast, Hersey et al. [22] found B7.1 mRNA expression in 50% of melanoma cells, although most melanoma cells did not express B7.1 protein on the membrane.

Prospects

In conclusion, we demonstrate a specific B7.1 neoexpression induced by γ-ray irradiation in tumor cells. This neoexpression of the B7.1 molecule could explain the irradiation-enhanced immunogenicity of many tumor cells, which may present the antigen and then directly prime the lymphocytes, provided that they express both B7.1 and MHC class I molecules [8]. Consistent with this idea, animals immunized with irradiated P815 or mhAT2 cells (MHC-class-I-positive, B7.1-positive) develop a long-lasting antitumor immunity [9] (and personal data). By contrast, irradiated AK7 cells (B7.1-positive but MHC-class-I-negative) or MCA101 cells (expressing low levels of MHC class I and no B7.1) are unable to induce and enhance systemic immunity [9].

It has recently been reported by Eton et al. [12] that UV-B irradiation of human melanoma cells induces expression of the B7.1 molecule, which is associated with tumor regression in some patients injected with UV-B-irradiated autologous melanoma cells. This further emphasizes the importance of the expression of B7 costimulatory molecules in tumor cells used as vaccines. Furthermore, our data suggest a possible mean of inducing B7.1 molecule expression in vivo, which could lead to new protocols, investigating the possible cooperation between tumor irradiation and immunotherapies.

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