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Changed Adhesion Molecule Profile of Ewing Tumor Cell Lines and Xenografts under the Influence of Ionizing Radiation

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Abstract. *Background: Adhesion molecules are involved in cell-cell and cell-matrix interactions and may be informative to characterize intercellular mechanisms of invasion and metastasis. This study was performed to characterize radiation-induced changes in the adhesion molecule profile of Ewing tumor subpopulations on a single cell level. Materials and Methods: In the present study, two Ewing tumors were characterized in vitro 4, 24 and 72 hours after radiation with 5 Gy and in vivo in a xenograft model 4, 6 and 15 days after radiation with 30 Gy, together with non-irradiated controls, by five parameter flow cytometry. Directly fluorescence-conjugated antibodies that were directed against adhesion molecules (LFA-1 (CD11a), HCAM (CD44), VLA-2 (CD49b), ICAM-1 (CD54), NCAM (CD56), LECAM-1 (CD62L) and CD86) were used. Annexin V and 7-AAD were used to characterize radiation-induced apoptosis. Results: Tumor cell subpopulations were identified by the expression of adhesion molecules, apoptotic markers and DNA content. Heterogeneous changes of the adhesion molecule profile were identified on tumor cell subpopulations after radiation. The expression of CD11a and CD62L correlated with the expression of apoptosis-associated markers. Conclusion: The changes of flow cytometric profile under radiation may potentially correlate with a changed metastatic potential of tumor cell subpopulations.*

Surface adhesion molecules play an important, but still not completely clarified, role in the process of metastasis. Cancer metastasis is a complex cascade of events that includes intravasation of tumor cells into the lymphatic or blood circulation, arrest in distant organs *via* interactions of tumor cells with the new organ environment, tumor growth, invasion and angiogenesis. Considerable research has been directed towards understanding molecular mechanisms involved in these different steps of the metastatic cascade (1-4). Specific cell surface proteins are involved in mediating adhesion of cells to each other and of cells to extracellular matrix structures. Adhesion molecules are classified into several groups that include integrins, cadherins, members of the immunoglobulin family and selectins. The adhesion molecules exhibit a broad range of structural and functional diversity. In malignant tumors expression of adhesion molecules correlates with metastatic potential (5-8) and tumor differentiation (9).

Moreover, it is postulated that adhesion molecules are involved in the apoptotic pathway (10-12). The role of programmed cell death (apoptosis) (13) in malignancies has not yet been clarified completely. Ionizing radiation has been shown to induce apoptosis (14-16) and the importance of this mechanism is under discussion. As an early marker of apoptosis the binding of Annexin V to phosphatidylserine can be used (17, 18).

Radiation-induced changes of adhesion molecule expression have been shown for normal tissues (19-22) and malignant tumors (23-26). Ewing tumors and related tumors express several different adhesion molecules. An involvement of integrin expression in the adhesion characteristic of tumor cells has been shown for these tumors (6).

Flow cytometry has become a powerful technique to characterize tumor cells at a single cell level and to identify intraindividual heterogeneity and subpopulations within one individual tumor. A simultaneous characterization of adhesion molecules, DNA content and apoptosis-associated markers can be performed at a single cell level. Besides standard one or two parameter DNA content analysis, several publications describe simultaneous analysis of immunophenotype and

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DNA content (27-32). Multiparametric DNA flow cytometry is therefore a powerful tool to characterize simultaneously the adhesion molecule profile, the DNA content and apoptosis-associated markers (33,34) at a single cell level.

The aim of this study was to characterize Ewing tumor cells under the influence of ionizing radiation. Molecules that are potentially involved in tumor invasion, metastasis and apoptosis were chosen as target antigens.

Materials and Methods

Two Ewing tumor cell lines (ET-1 and RM-82) were characterized *in vitro* and in a xenograft mouse model under the influence of radiation.

Source of tumor cell lines. ET-1 was derived from a primary tumor (humerus) characterized as a malignant peripheral neuroectodermal tumor (PNET) (35). The cell line RM-82 originated from a primary Ewing's sarcoma of the femur (36).

Cell culture. Cells were grown in collagen-coated culture flasks in RPMI 1640 medium (supplemented with 10% fetal calf serum (FCS)) (Gibco Invitrogen Corporation, Karlsruhe, Germany) and maintained in a humidified incubator at 37˚C and 5% CO_2. All cells tested negative for mycoplasma.

Xenograft mouse model. For the xenograft experiments, NMRI athymic nude mice (nu/nu) were used (37,38). $5x10^6$ cells were transplanted subcutaneously in a 250µl suspension (RPMI 1640 medium). Solid tumor samples were examined by light microscopy, immunohistochemistry and flow cytometry to prove the human Ewing tumor family origin after the engraftment.

Irradiation. Cells and solid tumors were irradiated with 6MV photons from a linear accelerator with a dose rate of 4.8 Gy/min at room temperature. The cell lines were irradiated *in vitro* with 5 Gy and analyzed after 4, 24 and 72 hours. Xenograft tumors were characterized 4, 6 and 15 days after a single radiation fraction with 30 Gy. Non-irradiated samples were used as controls. A tissue equivalent material was used to reduce the build-up effect.

The radiation doses and points of time of characterization after radiation were chosen depending on the experimental model used. Cell culture experiments were used for short time experiments and cells were analyzed between 4 to 72 hours after radiation with 5 Gy. The *in vivo* model was chosen for long time analysis and cells were analyzed between 4 to 15 days after radiation with 30 Gy. Although radiation doses were different, this set-up was chosen because of the practicality of both systems.

Cell preparation, immunophenotyping, Annexin V and DNA staining. For the *in vitro* characterization of cell lines, the cultured cells were disaggregated with phosphate-buffered saline (PBS) (Gibco Invitrogen Corporation) containing 0.5 mM EDTA (Sigma, Taufkirchen, Germany).

For *in vivo* analyses of xenograft tumors, a single cell suspension adequate for flow cytometric analyses had to be prepared. The tumor was explanted and mechanically disaggregated, filtered through a 35-µm mesh and resuspended in 300 µl PBS (containing 1% bovine serum albumin (BSA) (Sigma)) as previously described (32).

For simultaneous immunophenotyping and DNA staining, the cell number was adjusted between $2x10^5$ and $1x10^6$ cells in 100 µl PBS/BSA. Monoclonal antibodies conjugated directly to fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were added to the cell suspension. Aliquots were incubated at 4˚C for at least 20 minutes in the dark. All antibodies belonged to the subclass IgG1 and were directed against cell adhesion molecules (HCAM (CD44) (Becton Dickinson (BD), Heidelberg, Germany), LFA-1 (CD11a), ICAM-1 (CD54), NCAM (CD56), LECAM-1 (CD62L) (all BD Pharmingen) and CD86 (Genzyme, Rüsselsheim, Germany)).

For the identification of human Ewing tumor cells, antibodies against HLA-ABC (BD Pharmingen) and against the Ewing tumor specific marker CD99 (Serotec, Eching, Germany) (35) were used. For the detection of non-specific binding, isotype controls (IgG1 FITC / PE (BD)) were used. After incubation, cells were washed once with PBS/BSA and then resuspended in 300 µl of cold 70% methanol for at least one hour. After fixation, the cell suspension was washed again with PBS/BSA and resuspended in 300 µl PBS/BSA. Five µl 7-aminoactinomycin D (7-AAD) (Sigma) at a concentration of 25 µg/ml were added and cells were incubated for at least 48 hours. An analysis of DNA content was also performed using 4', 6-diamidino-2-phenylindole (DAPI) (Serva, Heidelberg, Germany) and propidium iodide (PI) (Sigma) and compared with results obtained by 7-AAD.

For Annexin V (BD Pharmingen) and DNA staining, cells were resuspended in Annexin V binding buffer (BD Pharmingen) after cell surface staining with antibodies directed against CD11a and CD62L. A 5 µl PE-conjugated Annexin V and a 5 µl 7-AAD solution at a concentration of 25 µg/ml was added and incubated for 15 minutes at room temperature. Cells were acquired for analysis within one hour after staining.

Flow cytometric acquisition and analysis. Samples were analyzed on a flow cytometer (FACSCalibur, BD). Electronic compensation was used to remove spectral overlap. Monoclonal antibody staining was detected using log amplification. Simultaneous DNA staining was detected using fluorescence 3 (FL3) in linear mode. In the case of simultaneous Annexin V and DNA staining the DNA signal was detected on a log scale. Forward and side scatter (FSC and SSC) signals were acquired in linear mode. FL1 and FL2 photomultiplier (PMT) voltage settings were set using unstained and isotype samples. The FL3 PMT voltage was set according to the G0/G1 peak of the dominant tumor cell population. Besides FL3 pulse height signals, width and area signals were detected to exclude doublets. Dual thresholding using FSC and FL3 signals was set to exclude debris without excluding populations of interest. No threshold on FL3 was used in the case of Annexin V and DNA staining and the FL3 PMT voltage was set according to a non-apoptotic cell population. Data was analyzed using Paint-a-Gate and Cell Quest Pro software (BD). Debris and unclassifiable events were not painted.

Results

The adhesion molecule profile, based on the expression of CD11a, CD44, CD49b, CD54, CD56, CD62L and CD86, was characterized for both tumor cell lines *in vitro* and in a xenograft model *in vivo*. The adhesion molecule profile showed a clear inter- as well as intraindividual heterogeneity before and after radiation. This individual characteristic profile is shown for both tumors *in vitro* before radiation in Figure 1.

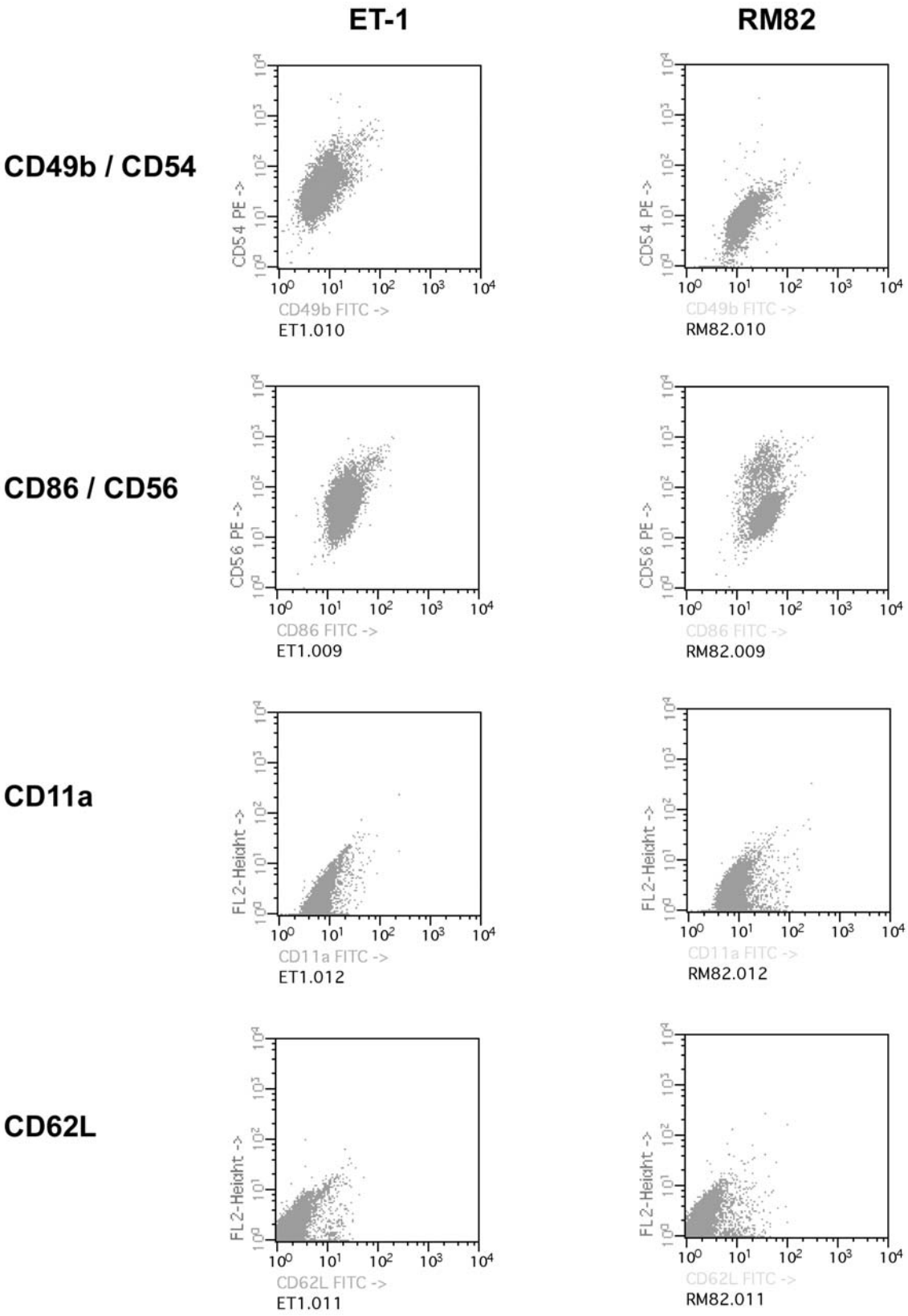

Figure 1. *In vitro characterization of adhesion molecule profile of the Ewing tumor cell lines ET-1 and RM-82 characterized by the expression of CD11a, CD49b, CD54, CD56 and CD62L. Both cell lines show an intra- and interindividual heterogeneous expression of adhesion molecules.*

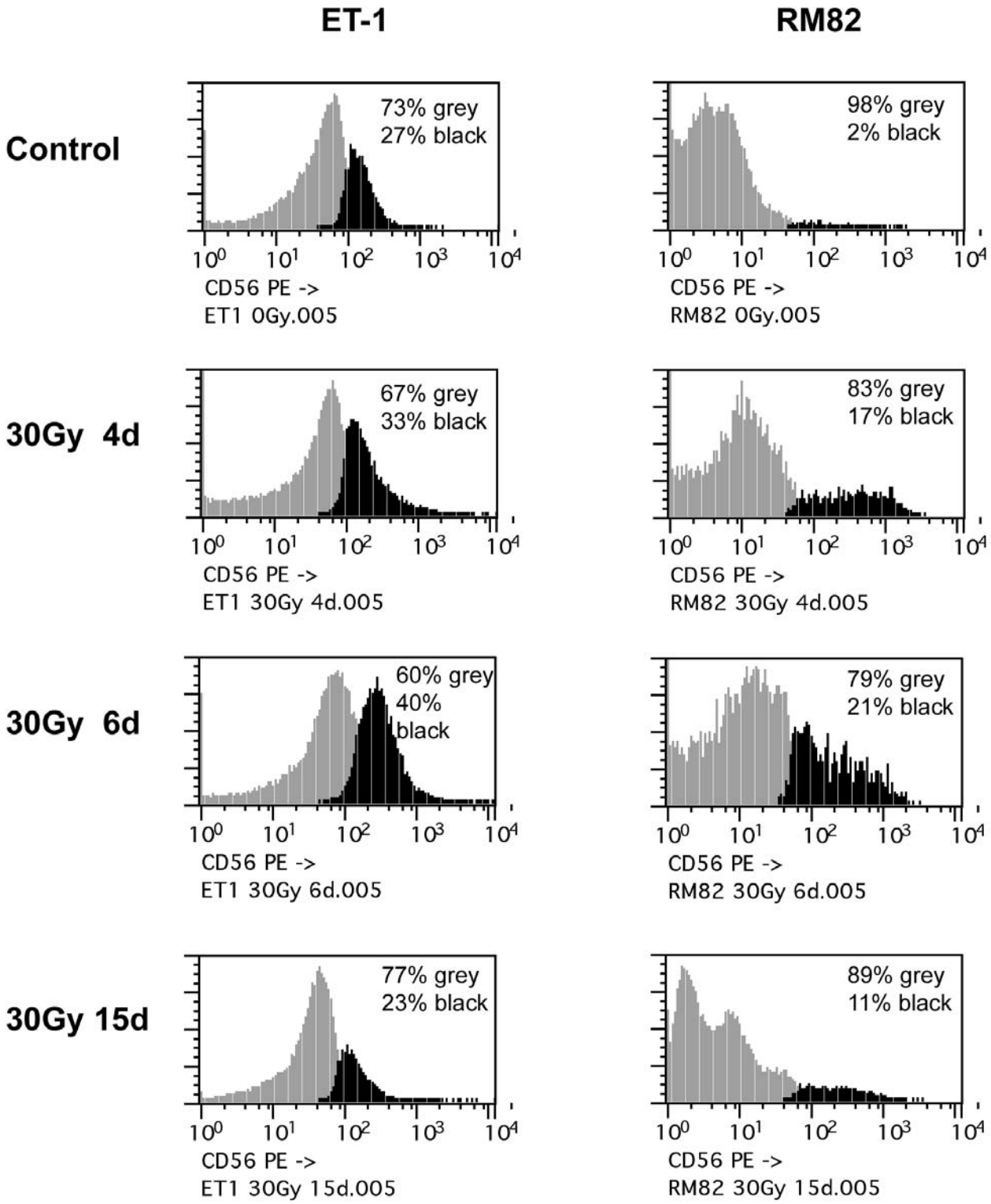

Figure 2. *Characterization of CD56 expression on tumor cells of ET-1 and RM-82 before radiation as well as 4, 6 and 15 days after radiation with 30 Gy in a xenograft model. Two subpopulations of cells can be distinguished by the expression of CD56, painted grey and black. After radiation an increase of CD56-positive cells is shown, followed by a decrease 15 days after radiation. The percentage of both identified subpopulations is given.*

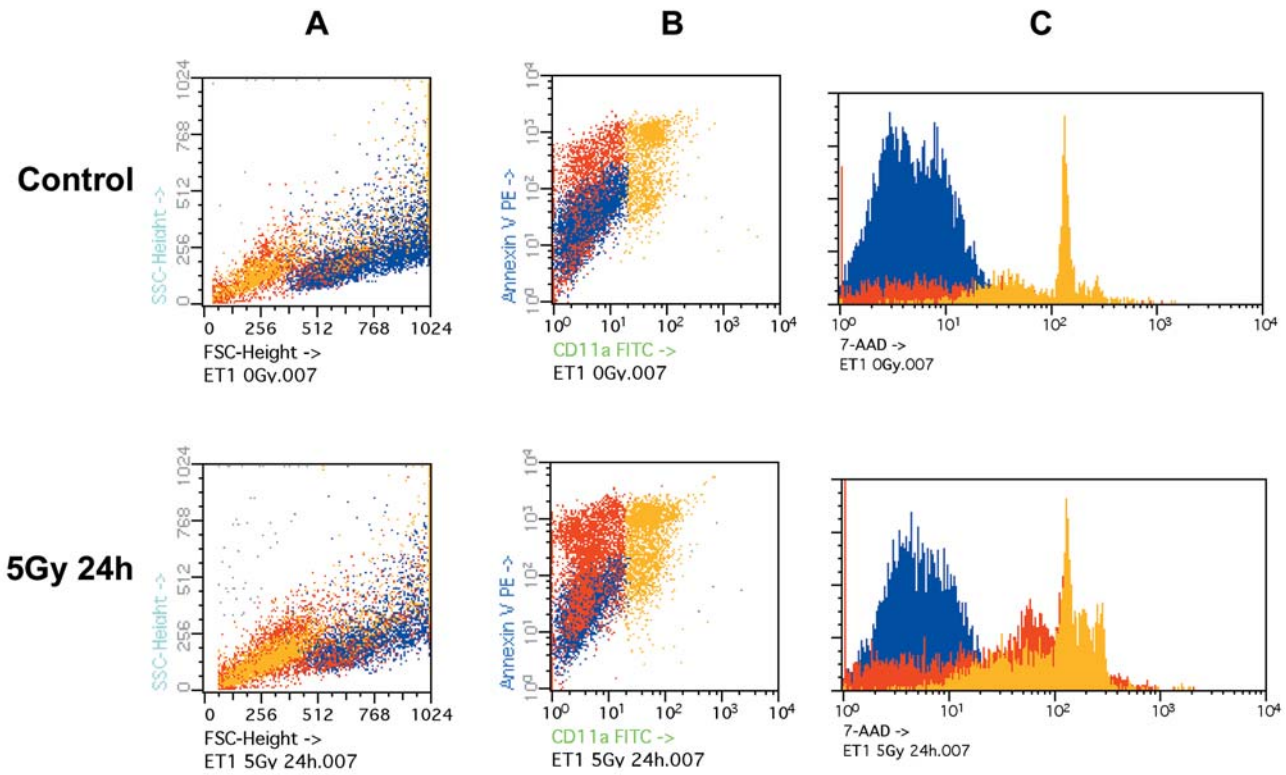

Figure 3. *Simultaneous analysis of light scatter profile, CD11a and Annexin V expression together with stainability for 7-AAD, used as apoptosis-associated marker. In this figure cells of ET-1 are characterized in vitro before and 24 hours after radiation with 5 Gy. Non apoptotic cells are painted blue and are characterized by light scatter profile, a dim expression of Annexin V and non-stainability for 7-AAD. Annexin-positive cells are painted red and yellow and are characterized by decreased light scatter parameters and stainability for 7-AAD. These cells have to be classified as predominant early apoptotic cells. Annexin-positive cells are distinguished by the expression of CD11a. Negative cells are painted red, CD11a-positive cells are painted yellow. The percentage of each identified subpopulation is given. After radiation the percentage of non apoptotic cells (painted blue) decreases and the percentage of apoptotic cells (painted red and yellow) increases. CD11a expression is clearly associated with Annexin V expression, however Annexin V-positive and CD11a-negative cells are detectable as well. Among the Annexin V-positive cells several subpopulations can be detected (not distinguished).*

Both cell lines were positive for CD54 and CD56 *in vitro* before radiation. An up-regulation of CD54 and CD56 was detectable on a subpopulation of cells of ET-1 72 hours after radiation with 5 Gy *in vitro*. The profile of RM-82 remained unchanged compared to the non-irradiated control. Cells of ET-1 and RM-82 showed a comparable CD54 profile *in vitro* and *in vivo*. In the case of CD56 the xenograft tumor ET-1 tended to a higher expression of CD56 on a subpopulation of cells, while the xenograft RM-82 tended to a lower expression on a subpopulation of cells. In the xenograft model, ET-1 and RM-82 showed a time-dependent up-regulation on a subpopulation of cells of CD56 4 and 6 days after radiation with 30 Gy, followed by a decrease 15 days after radiation (Figure 2). The changes of the expression of CD54 on ET-1 cells were comparable after radiation with those in the xenograft model. Cells of RM-82 showed no changed expression.

CD44 was negative on cells of RM-82 *in vitro* and *in vivo*. CD44 was dim-positive on a subpopulation of cells of ET-1 *in vitro* and *in vivo* and in both cases unchanged after radiation.

All cells of ET-1 and RM-82 were dim-positive for CD49b *in vitro* and *in vivo* without any changes *in vitro* after radiation. An up-regulation of CD49b on a small subpopulation of cells of both tumors *in vivo* was detectable up to 15 days after radiation.

CD86 was positive on all cells of ET-1 and RM-82 *in vitro* and *in vivo*. No changes of the CD86 profile were identified after radiation.

The majority of cells of both tumors were negative for CD11a and CD62L *in vitro*; a minor subpopulation was positive before radiation. A time-dependent up-regulation of CD11a and CD62L was observed on a subpopulation of cells of both cell lines 24 hours after radiation (Figure 3b). It remained up-regulated at 72 hours after radiation with a tendency to a decreased expression. Before radiation, xenograft tumor cells showed a profile comparable to the *in vitro* profile. No enhanced expression of either antigen was observed 4, 6 or 15 days after radiation.

The cell lines ET-1 and RM-82 were characterized according to the binding of Annexin V and stainability for 7-AAD. Simultaneous characterization of the adhesion molecules CD11a and CD62L was performed *in vitro*. According to Annexin V binding and stainability for 7-AAD different subpopulations were characterized before as well as 4, 24 and 72 hours after radiation with 5 Gy. These identified subpopulations represent the different stages of the apoptotic pathway. Simultaneous characterization of the cell surface adhesion molecule profile was performed according to the expression of CD11a and CD62L. The expression of these adhesion molecules was associated with the binding of Annexin V. In all cases two subpopulations of the Annexin-positive cells were positive for CD11a and CD62L and were distinguished by a difference in the intensity of Annexin V binding. Moreover, a third Annexin V-positive and CD11a/CD62L-negative subpopulation was identified. This is shown in Figure 3 for ET-1 cells before and 24 hours after radiation with 5 Gy. Light scatter profile (Figure 3A), expression of CD11a as well as Annexin V (Figure 3B) and stainability for 7-AAD (Figure 3C) are characterized simultaneously and different subpopulations are distinguished. The Annexin V-positive subpopulations increased compared to the non-irradiated control sample after radiation.

Discussion

Characterization of markers involved in cell-cell and cell-matrix interactions like adhesion molecules, as well as markers associated with apoptosis like Annexin V and stainability for DNA dyes, may be important to understand intercellular mechanisms related to metastasis, radiation response and therapy failure.

The aim of our study was to characterize tumor subpopulations and to detect tumor cell heterogeneity before and changes of profile after radiation, by multiparametric flow cytometry at a single cell level, by the simultaneous characterization of markers potentially associated with intercellular interactions, like metastasis and apoptosis. Two Ewing tumors were immunophenotyped *in vitro,* respectively, in a xenograft mouse model before and at different points of time after radiation. These results may be the basis for further studies examining the role of adhesion molecules in the processes of metastasis and apoptosis.

The expression of the adhesion molecules CD44, CD49b and CD86 as analyzed in this study was shown to be non-informative for the characterization of Ewing tumor cells under the influence of ionizing radiation. No relevant changes of profile were detectable. However CD49b showed radiation-induced changes in a colorectal tumor cell line (25).

The intercellular adhesion molecule ICAM-1 (CD54) (39) and the neural cell adhesion molecule NCAM (CD56) (40) belong to the immunoglobulin supergene family. Ewing tumors were tested partially positive for the expression of CD56 by immunohistochemical and flow cytometric methods (41). Neuroblastomas and related tumors showed highly variable phenotypes based on the expression of CD54 and CD56. These adhesion molecules were also associated with tumor differentiation (9).

The tumor cells analyzed in the present study showed a heterogeneous profile of CD54 and CD56 expression *in vitro* and *in vivo* after radiation. This indicates an interindividual heterogeneous response to radiation. This heterogeneity is also detectable within one individual tumor because the observed up-regulation of these adhesion molecules is only detectable on a subpopulation of tumor cells. This indicates complex individual intercellular mechanisms in the expression of these adhesion molecules.

The up-regulation of the observed adhesion molecules CD56 and CD54 is in concordance with other authors. An enhancement of the expression of ICAM-1 (CD54) after radiation is described for normal tissues (19-22) and for malignancies (24).

The neuronal cell adhesion molecule NCAM (CD56) is involved in neuronal homotypic cell adhesion and in cell differentiation during embryogenesis. For malignant melanoma Geertsen *et al.* (8) described NCAM as an immunoregulating molecule involved in the formation of CNS metastases. For glioma cell lines, an up-regulation of CD56 is described after radiation (42).

The leukocyte function-associated molecule 1 LFA-1 (CD11a) belongs to the integrin family and is a membrane glycoprotein that functions in cell-cell adhesions by heterophilic interaction with the intercellular adhesion molecule-1 (ICAM-1) (43). In this study a time-dependent up-regulation of CD11a was shown *in vitro* for both analyzed cell lines up to 72 hours after radiation. This effect was no longer detectable four days *in vivo* after radiation. The expression of this adhesion molecule was associated with Annexin V expression, indicating that CD11a expression is either influenced by or mediates the apoptotic process, which was shown also by Zhou *et al.* (10). An increased expression of CD11a and CD54 was observed on normal human alveolar macrophages after irradiation. The authors concluded that these adhesion molecules play a part in the development of radiation-induced lung injury (21). The involvement of specific integrins in the adhesion of primary and metastatic Ewing's Sarcomas was shown by van Valen *et al.* (6).

CD62L belongs to the selectin family (44). L-selectin is a prerequisite for leukocyte adhesion to endothelial cells of blood vessels and consequently for transmigration. Kern *et al.* observed a loss of CD62L on peripheral-blood-mononuclear-cells after low-dose gamma radiotherapy (11) and on apoptotic lymphocytes after UVB-radiation (12). They concluded that shedding of CD62L in early leukocyte

apoptosis is an active process and important for the regulation of inflammation. Little is known about the expression of CD62L on tumor cells under the influence of radiation.

In the present study a time-dependent up-regulation of CD62L was shown corresponding with the results obtained for CD11a. The observed coexpression of the apoptosis-associated marker Annexin V, CD11a and CD62L on the same subpopulation of cells in both analyzed tumors makes the involvement of these adhesion molecules in apoptotic mechanisms likely. The potential involvement of these adhesion molecules in the clearance process of apoptotic or dying cells could be a possible explanation for the observed phenomenon.

Functional studies with different subpopulations have to be performed to examine whether the observed changes of the adhesion molecule profile have an influence on the metastatic potential of tumor cell subpopulations.

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References

1 Behrens J, Frixen U, Schipper J, Weidner M and Birchmeier W: Cell adhesion in invasion and metastasis. Semin Cell Biol *3*: 169-178, 1992.

2 Zetter BR: Adhesion molecules in tumor metastasis. Sem Cancer Biol *4*: 219-229, 1993.

3 Clezardin P: Recent insights into the role of integrins in cancer metastasis. Cell Mol Life Sci *54*: 541-548,1998.

4 Petruzzelli L, Takami M and Humes HD: Structure and function of cell adhesion molecules. Am J Med *106*: 467-476, 1999.

5 Miele ME, Bennett F, Miller BE and Welch DR: Enhanced metastatic ability of TNF-a-treated malignant melanoma cells is reduced by Intercellular Adhesion Molecule-1 (ICAM-1, CD54) antisense oligonucleotides. Exp Cell Res *214*: 231-241, 1994.

6 van Valen F, Hanenberg H and Jürgens H: Expression of functional very late antigen alpha 1, 2, 6 integrins on Ewing's sarcoma and primitive peripheral neuroectodermal tumor cells and modulation by interferon gamma and tumor necrosis factor alpha. Eur J Cancer *30A*: 2119-2125, 1994.

7 Koga H, Naito S, Nakashima M, Hasegawa S, Watanabe T and Kumazawa J: A flow cytometric analysis of the expression of adhesion molecules on human renal cell carcinoma cells with different metastatic potentials. Eur Urol *31*: 86-91, 1997.

8 Geertsen R, Zenklusen R, Kamarashev J, Burg G and Dummer R: Inverse regulation of neuronal cellular adhesion molecule (NCAM) by IFN-gamma in melanoma cell cultures established from CNS lesions. Int J Cancer *83*: 135-140, 1999.

9 Gross N, Favre S, Beck D and Meyer M: Differentiation-related expression of adhesion molecules and receptors on human neuroblastoma tissues, cell lines and variants. Int J Cancer *52*: 85-91, 1992.

10 Zhou YW, Komada Y, Inaba H, Deguchi T, Sugiyama K, Azuma E and Sakurai M: Functional significance of adhesion molecules in FAS-dependent apoptotic cell death induced by Interleukin-2-activated T cells. Immunol Invest *27*: 309-322, 1998.

11 Kern PM, Keilholz L, Forster C, Hallmann R, Herrmann M and Seegenschmiedt MH: Low-dose radiotherapy selectively reduces adhesion of peripheral blood mononuclear cells to endothelium *in vitro*. Radiother Oncol *54*: 273-282, 2000.

12 Kern PM, Keilholz L, Forster C, Stach C, Beyer TD, Gaipl US, Kalden JR, Hallmann R and Herrmann M: UVB-irradiated T-cells undergoing apoptosis lose L-selectin by metalloprotease-mediated shedding. Int J Radiat Biol *76*: 1265-1271, 2000.

13 Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, Culhane AC and Williams GT: Apoptosis: molecular regulation of cell death. Eur J Bioch *236*: 1-26, 1996.

14 Abend M and van Beuningen D: Bedeutung apoptotischer Prozesse für die Strahlentherapie. Teil I. Strahlenther Onkol *174*: 156-166, 1998.

15 Abend M and van Beuningen D: Bedeutung apoptotischer Prozesse für die Strahlentherapie. Teil II. Strahlenther Onkol *174*: 212–216, 1998.

16 Dahm-Daphi J: p53: Biology and role for cellular radiosensitivity. Strahlenther Onkol *176*: 278–285, 2000.

17 Van Engeland M, Nieland LJW, Ramaekers FCS, Schutte B and Reutelingsperger CPM: Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry *31*: 1-9, 1998.

18 Bedner E, Li X, Gorczyca W, Melamed MR and Darzynkiewicz Z: Analysis of apoptosis by laser scanning cytometry. Cytometry *35*: 181-195, 1999.

19 Hong JH, Chiang CS, Campbell IL, Sun JR, Withers HR and McBride WH: Induction of acute phase gene expression by brain irradiation. Int J Radiat Oncol Biol Phys *33*: 619-626, 1995.

20 Hallahan DE and Virudachalam S: Ionizing radiation mediates expression of cell adhesion molecules in distinct histological patterns within the lung. Cancer Res *57*: 2096-2099, 1997.

21 Kawana A, Shioya S, Katoh H, Tsuji C, Tsuda M and Ohta Y: Expression of intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 on alveolar macrophages in the acute stage of radiation-induced lung injury in rats. Radiat Res *147*: 431-436, 1997.

22 Quarmby S, Hunter RD and Kumar S: Irradiation induced expression of CD31, ICAM-1 and VCAM-1 in human microvascular endothelial cells. Anticancer Res *20*: 3375-3382, 2000.

23 Santin AD, Rose GS, Hiserodt JC, Fruehauf J, Eck LM, Garcia RI, Schranz V, Disaia PJ, Pecorelli S and Granger GA: Effects of cytokines combined with high-dose gamma irradiation on the expression of major histocompatibility complex molecules and intercellular adhesion molecule-1 in human ovarian cancers. Int J Cancer *65*: 688-694, 1996.

24 Hareyama M, Imai K, Oouchi A, Takahashi H, Hinoda Y, Tsujisaki M, Adachi M, Shonai T, Sakata K and Morita K: The effect of radiation on the expression of intercellular adhesion molecule-1 of human adenocarcinoma cells. Int J Radiat Oncol Biol Phys *40*: 691-696, 1998.

25 Meineke V, Gilbertz KP, Schilperoort K, Cordes N, Sendler A, Moede T and van Beuningen D: Ionizing radiation modulates cell surface integrin expression and adhesion of COLO-320 cells to collagen and fibronectin *in vitro*. Strahlenther Onkol *178*: 709-714, 2002.

26 Cordes N, Blaese MA, Meineke V and Van Beuningen D: Ionizing radiation induces up-regulation of functional beta1-integrin in human lung tumour cell lines *in vitro*. Int J Radiat Biol *78*: 347-357, 2002.
27 Hollander Z and Loken MR: Simultaneous analysis of DNA content and surface antigens in human bone marrow. Cytometry *9*: 485-490, 1988.
28 Brons PP, Pennings AH, Haanen C, Wessels HM and Boezeman JB: Simultaneous measurement of DNA content and cell-surface immunofluorescence of human bone marrow cells using a single laser flow cytometer. Cytometry *11*: 837-844, 1990.
29 Schmid I, Uittenbogaart CH and Giorgi JV: A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. Cytometry *12*: 279-285,1990.
30 Stokke T, Holte H, Erikstein B, Davies CL, Funderud S and Stehen HB: Simultaneous assessment of chromatin structure, DNA content, and antigen expression by dual wavelength excitation flow cytometry. Cytometry *12*: 172-178,1991.
31 Corver WE, Bonsing BA, Abeln ECA, Vlak-Theil PM, Cornelisse CJ and Fleuren GJ: One-tube triple staining method for flow cytometric analysis of DNA ploidy and phenotypic heterogeneity of human solid tumors using single laser excitation. Cytometry *25*: 358-366,1996.
32 Könemann S, Schuck A, Malath J, Rupek T, Horn K, Baumann M, Vormoor J, Rübe C and Willich N: Cell heterogeneity and subpopulations in solid tumors characterized by simultaneous immunophenotyping and DNA content analysis. Cytometry *41*: 172-177, 2000.
33 Bacso Z and Eliason JF: Measurement of DNA damage associated with apoptosis by laser scanning cytometry. Cytometry *45*: 180-186, 2001.
34 Rasola A and Geuna M: A flow cytometry assay simultaneously detects independent apoptotic parameters. Cytometry *45*: 151-157, 2001.
35 Ambros IM, Ambros PF, Strehl S, Kovar H, Gadner H and Salabendzer-Kuntschick M: MIC2 is a specific marker of Ewing's sarcoma and peripheral primitive neuroectodermal tumors. Evidence for a common histogenesis of Ewing's sarcoma and peripheral primitive neuroectodermal tumors from MIC2 expression and specific chromosome aberration. Cancer *67*: 1886-1893, 1991.
36 van Valen F, Winkelmann W, Burdach S, Gobel U and Jürgens H: Interferon gamma and tumor necrosis factor induce a synergistic antiproliferative response in human Ewing sarcoma cells *in vivo*. J Cancer Res Clin Oncol *119*: 615-621, 1993.
37 Floersheim GL: Comparative growth of human tumors in pharmacologically immunosuppressed, immune-deprived, cyclosporin-A treated and nude mice. Eur J Cancer Clin Oncol *18*: 589-594, 1982.
38 Phillips RA, Jewett MAS and Gallie BL: Growth of human tumors in immune-deficient scid mice and nude mice. Curr Top Microbiol Immun *152*: 259-263, 1989.
39 Simmons D, Makgoba MW and Seed B: ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature *331*: 624-627, 1988.
40 Cunningham BA, Hemperly JJ, Murray BA, Prediger EA, Brackenbury R and Edelman GM: Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science *236*: 799-806,1987.
41 Gardner LJ, Polski JM, Fallon R and Dunphy CH: Identification of CD56 and CD57 by flow cytometry in Ewing's sarcoma or primitive neuroectodermal tumor. Virch Arch *433*: 35-40, 1998.
42 Yamanaka R, Tanaka R and Yoshida S: Effects of irradiation on the expression of the adhesion molecules (NCAM, ICAM-1) by glioma cell lines. Neurol Med Chir *33*: 749-752, 1993.
43 Larson RS, Corbi AL, Berman L and Springer T: Primary structure of the leukocyte function-associated molecule-1 alpha subunit: an integrin with an embedded domain defining a protein superfamily. J Cell Biol *108*: 703-712, 1989.
44 Tedder TF, Isaacs CM, Ernst TJ, Demetri GD, Adler DA and Disteche CM: Isolation and chromosomal localization of cDNAs encoding a novel human lymphocyte cell surface molecule, LAM-1. Homology with the mouse lymphocyte homing receptor and other human adhesion proteins. J Exp Med *170*: 123-133, 1989.

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