Published in final edited form as:

Radiat Res. 2010 April ; 173(4): 418–425. doi:10.1667/RR1860.1.

Up-regulation of the Pro-inflammatory Chemokine CXCL16 is a Common Response of Tumor Cells to Ionizing Radiation

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Abstract

We recently showed that mouse and human breast carcinoma cells respond to ionizing radiation therapy by up-regulating the expression and release of the pro-inflammatory chemokine CXCL16, which binds to the CXCR6 receptor expressed by activated T cells. Enhanced recruitment of activated T cells to irradiated mouse 4T1 breast tumors was mediated largely by CXCL16 and was correlated with tumor inhibition in mice treated with the combination of local radiation and immunotherapy. In this study, the expression of CXCL16 and its modulation by radiation were analyzed in mouse melanoma B16/F10, fibrosarcoma MC57, colon carcinoma MCA38, and prostate carcinoma TRAMP-C1 cells. Only TRAMP-C1 cells showed detectable expression of CXCL16, although the level was lower than in 4T1 and 67NR breast carcinoma cells. Ionizing radiation up-regulated CXCL16 expression in all cells except B16/F10, but only TRAMP-C1, 67NR and 4T1 cells released the soluble chemokine in significant quantities. The metalloproteinases ADAM10 and ADAM17, which are responsible for cleaving the chemokine domain from the CXCL16 transmembrane form, were expressed in all cells. Overall, our data indicate that up-regulation of CXCL16 is a common response of tumor cells to radiation, and they have important implications for the use of local radiotherapy in combination with immunotherapy.

INTRODUCTION

Chemokines regulate processes such as cell migration, invasion, interaction with the endothelium and extracellular matrix, and survival. It is therefore intriguing that many cells acquire the expression of chemokine receptors and/or secrete chemokines when they become malignant [reviewed in ref. (1)]. Importantly, the production of chemokines by cancer cells has been shown to influence the degree and phenotype of the inflammatory infiltrate, mostly resulting in the recruitment of leukocytes with immunosuppressive and pro-angiogenic functions (1,2).

CXCL16 is one of only two chemokines that are expressed as transmembrane molecules. The chemokine domain can be cleaved from the cell surface by the activity of the disintegrin-like metalloproteinase (MPase) ADAM10 (3,4). CXCR6 is the only known receptor for CXCL16 and is expressed on subsets of effector T cells, natural killer (NK) cells, NKT cells and plasma cells (5–8). Soluble CXCL16 induces chemotaxis of CXCR6⁺ T and plasma cells. The transmembrane form can mediate adhesion to CXCR6⁺ cells as well as function as a scavenger receptor for oxidized low-density lipoprotein, phosphatidylserine and dextran sulfate (9). Until recently, CXCL16 was known to be expressed mostly by

dendritic cells, macrophages and endothelial cells and to be up-regulated during inflammation in different organs, where it mediates recruitment of activated CD8⁺ T cells (10,11). We and others have recently shown that CXCL16 is also expressed in breast carcinoma (12,13), and its expression in a few other malignancies has been reported as well (14–17).

The function of CXCL16 expressed by cancer cells remains incompletely understood. Data for renal cell carcinoma suggest that it functions as a tumor suppressor gene, inhibiting the growth or migration of the tumor cells (16,18). In contrast, in pancreatic and prostate carcinoma cells, CXCL16 was shown to enhance tumor invasiveness (15,19). In most cases, the effects of CXCL16 were mediated by binding to the CXCR6 receptor expressed by the same cancer cells in an autocrine fashion. The form of CXCL16 was critical in determining inhibition (transmembrane form) or stimulation (soluble form) of cancer cell growth (13). Other data suggest that the effects of CXCL16 produced by cancer cells are mediated by its interaction with the host immune system. In colorectal carcinoma patients, higher levels of CXCL16 expression in the tumor cells correlated with increased numbers of tumor-infiltrating T cells and with a better prognosis (14).

We recently showed in the 4T1 mouse breast cancer model that treatment with ionizing radiation up-regulated the expression and release of CXCL16 by the tumor cells markedly, resulting in increased migration of activated CD8⁺ T cells toward the irradiated 4T1 cells *in vitro* and *in vivo* (12). CXCR6 was expressed by most of the CD8⁺ T cells infiltrating 4T1 tumors in mice treated with local radiotherapy and anti-CTLA-4 antibody, a treatment that elicits therapeutically effective CD8⁺ anti-tumor T cells (20). Mice deficient in CXCR6 expression showed decreased CD8⁺ T-cell infiltration in the tumors and decreased the ability to control tumor growth after treatment (12).

Overall, the data described above suggest the possibility that higher CXCL16 expression by the tumor cells may be beneficial if anti-tumor T cells are present in the host but may drive tumor growth in the absence of an anti-tumor immune response. Therefore, it is important to determine whether up-regulation of CXCL16 expression in tumors is a common effect of radiotherapy. Here we show that tumors of different tissue origin up-regulate CXCL16 expression in response to radiation, supporting the use of immunotherapy in combination with radiotherapy.

MATERIALS AND METHODS

Tumor Cell Lines

4T1 and 67NR are BALB/c mouse-derived breast carcinoma cell lines (21). The B16/F10 melanoma (22), MC57 fibrosarcoma (23) and MCA38 colon carcinoma (24) cell lines were derived from C57BL/6 mice. All cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5×10^{-5} M 2-mercapthoethanol, and 10% FBS (Gemini Bio-Products Woodland, CA) (complete medium). Cells of the TRAMP-C1 prostate carcinoma cell line (25) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured as recommended by ATCC in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.005 mg/ml bovine insulin, 10 nM dehydroisoandrosterone, 1.5 g/liter sodium bicarbonate, 5% FBS and 5% Nu-Serum IV.

Irradiation

Cells were irradiated with a Gammacell 1000 ¹³⁷Cs irradiator (Atomic Energy of Canada Limited) at dose rate of 2.78 Gy/min.

RT-PCR and Real-Time PCR

Total RNA was prepared from cells at different times after 12 Gy irradiation or mock treatment using RNeasy (Qiagen) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase (Promega) before PCR reaction.

For RT-PCR, RNA was used for cDNA synthesis performed with an omniscrypt RT kit (Qiagen) with Oligo-dT primers (Qiagen). The primers for PCR for CXCL16 (416 bp) were forward 5'-GCT TTG GAC CCT TGT CTC TTG C- 3', reverse 5'-GTG CTG AGT GCT CTG ACT ATG TGC- 3'; β -Actin (188 bp): forward 5'-AGG TGA CAG CAT TGC TTC TG- 3', reverse 5'-GCT GCC TCA ACA CCT CAA C- 3'. β -Actin was used as an internal control for equal loading, and both primers were added to the same tube to perform PCR. The primers for ADAM10 (120 bp) were forward 5'-AGC AAC ATC TGG GGA CAA AC- 3', reverse 5'-TGG CCA GAT TCA ACA AAA CA- 3'; the primers for ADAM17 (200 bp) were forward 5'-GTA CGT CGA TGC AGA GCA AA- 3', reverse 5'-AAA CCA GAA CAG ACC CAA CG- 3'. Different primers were used when β -actin was used as the control for ADAM10 and ADAM17 to obtain a PCR product of a specific size (540 bp): forward 5'-GTG GGC CGC TCT AGG CAC CAA- 3', reverse 5'-CTC TTT GAT GTC ACG CAC GAT TTC- 3'. Densitometric analysis was performed, and the ratio of each gene product to β -actin was calculated.

Real-time PCR was performed using an iCycler (Bio-Rad). An RT² First Strand Kit (SABiosciences) was used to synthesize cDNA followed by real-time RT-PCR with RT2 Real-Time TMSYBR Green/Fluorescein PCR Master Mix according to manufacturer's protocol. Samples were normalized to eIF4G II (eukaryotic translation initiation factor 4G II), and expression on untreated cells was assigned a relative value of 1.0. The primers used in this study were catalog no. PPM03775A for CXCL16 and catalog no. PPM36187A for eIF4G II (SABiosciences). Data were analyzed with Bio-Rad iCycler IQ software. To calculate the relative induction of chemokines, the $2^{(-\Delta\Delta Ct)}$ method was used.

Immunofluorescence Staining

Cells (2×10^5 /well) were plated in six-well plates 1 day before the experiment. The next day, the medium was replaced with 2 ml of 1% FBS DMEM containing 20 μ M BB-94 (Batimastat) or vehicle alone (DMSO). After 2 h incubation, the cells were harvested with 0.04% EDTA in PBS, washed and stained with goat anti-mouse CXCL16 antibody (4 μ l/ml) (R&D Systems) followed by FITC-donkey anti-goat IgG antibody from Jackson ImmunoResearch (West Grove, PA) diluted at 1:200. Cells were fixed and analyzed using an LSR II flow cytometer (Becton Dickinson) and FlowJo version 6.4.4 (Tree Star, Ashland, OR).

Measurement of Soluble CXCL16

Mouse carcinoma cells were plated at 1×10^4 cells/well in duplicate wells of a 96-well plate. Seventy-two hours after irradiation or mock treatment, the medium was changed to DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1% FBS. Some wells contained 20 μ M BB-94 (Batimastat). Released CXCL16 was measured in supernatants after 4 h by ELISA (RayBiotech, Norcross, GA).

Statistical Methods

An unpaired Student's *t* test was used to evaluate *P* values using data from three independent real-time RT-PCR experiments, each performed in duplicate. All reported *P* values are two-sided and were declared significant at the 5% level.

RESULTS

Expression of CXCL16 in Mouse Cancer Cells of Different Tissue Origin

We have recently showed that CXCL16 was expressed in three out of five mouse breast carcinoma lines of differing metastatic potential and in four out of four human cell lines, one derived from benign breast epithelium and three from primary breast cancers of different stage (12). To determine whether expression of CXCL16 is common in tumors derived from tissues other than the breast, we tested four lines that are widely used as mouse models of human neoplasms. Cells of the two mouse breast cancer cell lines, 4T1 and 67NR, were used as positive controls. CXCL16 mRNA was detected in the prostate carcinoma TRAMP-C1 cells, whereas the B16/F10 melanoma, MC57 fibrosarcoma and MCA38 colon carcinoma cells were negative as determined by RT-PCR (Fig. 1A). Relative to β -actin expression, TRAMP-C1 cells showed lower levels of CXCL16 than 4T1 and 67NR cells (Fig. 1B).

Next, expression of CXCL16 transmembrane form was tested by immunofluorescence staining and flow cytometry. CXCL16 surface expression was detectable only in cells preincubated for 2 h with the MPase inhibitor BB-94, suggesting that in TRAMP-C1 and 67NR cells, as in 4T1 cells (12), CXCL16 is rapidly shed in the soluble form by MPase-mediated cleavage and does not accumulate on the cell surface (Fig. 2). No staining for CXCL16 was seen in B16/F10, MC57 and MCA38 cells (data not shown).

Up-regulation of CXCL16 Expression and Release by Ionizing Radiation

Ionizing radiation has been shown to modulate the expression of many cell surface receptors on tumor cells (26). We reported the enhanced expression and release of CXCL16 in all mouse and human breast cancer cells tested after irradiation (12). Up-regulation of CXCL16 was dose-dependent and peaked at 6 Gy for 67NR cells, whereas for the other breast cancer cells it continued to increase until at least 12 Gy [ref. (12) and data not shown]. Therefore, 12 Gy was chosen as the test dose for evaluation of CXCL16 induction.

Baseline expression of CXCL16 in mock-irradiated cells was detected by real-time quantitative PCR in B16/F10, MC57 and MCA38 cells, which did not have a visible band by RT-PCR. Statistically significant ($P < 0.05$) induction of CXCL16 mRNA ranging from two- to threefold (TRAMP-C1 and MC57) to over tenfold (67NR) increases from baseline levels was seen in all cells except B16/F10 (Fig. 3). In 67NR and MCA38 cells, a small but significant increase was evident at 24 h, whereas in MC57 and TRAMP-C1 cells it was delayed. However, in all cells, the magnitude of the increase was greatest at 72 h. In 4T1 cells, CXCL16 mRNA showed a four- to fivefold increase in induction, peaking at 48 h postirradiation (data not shown), confirming our previous results (12).

To determine whether the radiation-mediated induction of CXCL16 mRNA resulted in increased release of the soluble chemokine, CXCL16 was measured by ELISA in supernatants of cells incubated for 4 h in the presence or absence of the MPase inhibitor BB-94 72 h after mock treatment or 12 Gy irradiation. Consistent with the very low basal levels of mRNA expression, CXCL16 was not detectable in the supernatants of nonirradiated MCA38 cells, whereas the average five-fold enhancement of CXCL16 expression in irradiated cells resulted in low but detectable CXCL16 release (Fig. 4). In contrast, the lesser enhancement (average of 2.5-fold) induced by radiation in MC57 cells was not sufficient to result in the release of detectable levels of CXCL16 by these cells (data not shown). Interestingly, despite the higher baseline CXCL16 expression in 67NR cells (Fig. 1), nonirradiated TRAMP-C1 and 67NR cells released similar amounts of CXCL16 (Fig. 4). In addition, the radiation-induced enhancement in protein released by TRAMP-C1 and 67NR cells was comparable (Fig. 4), whereas the relative up-regulation of CXCL16

mRNA expression by radiation was markedly higher in 67NR cells (Fig. 3), suggesting that additional mechanisms may regulate the release of CXCL16 by irradiated cancer cells. Among all cells tested, 4T1 released the highest amounts of CXCL16 after irradiation (Fig. 4).

In all cells, the baseline as well as the radiation-induced release of CXCL16 was inhibited by BB-94, indicating that it was mediated by an MPase. Two MPases, ADAM-10 and ADAM-17, have been implicated in the constitutive and inducible release of CXCL16, respectively (3,27). To determine whether differences in the expression of ADAM-10 and ADAM-17 could explain in part the differences in the amount of CXCL16 released by the cancer cells, expression of these MPases was tested by RT-PCR. ADAM-10 and ADAM-17 were expressed in all cells and were not modulated by radiation (Fig. 5). The relative levels of expression of the MPases in different cells did not correlate with the levels of released CXCL16; for example, 4T1 cells had lower expression of ADAM-10 and ADAM-17 compared to 67NR cells but released more CXCL16 (Fig. 4 and Fig. 5), suggesting that levels of expression do not reflect the activity of the MPases, which is regulated by additional mechanisms (28).

Overall, data demonstrate that cancer cells of different histological derivation respond to ionizing radiation by up-regulating CXCL16 gene expression. However, the levels released vary widely between cells, with as much as a thousandfold difference in the amount of CXCL16 released by the lowest (MCA38) and the highest (4T1) producers.

DISCUSSION

In this study, we show for the first time that mouse cancer cells that are commonly used as models of prostate and colorectal carcinoma and fibrosarcoma up-regulate CXCL16 expression in response to ionizing radiation, whereas the B16/F10 melanoma does not.

The baseline expression of CXCL16 in untreated cancer cells was generally low and only the breast 4T1 and 67NR and prostate TRAMP-C1 carcinoma cells released detectable amounts of soluble CXCL16. Cell surface expression of CXCL16 was seen only in the presence of an inhibitor of MPase activity, indicating that cancer cells readily shed the soluble chemokine (Fig. 2 and Fig. 4). These data are consistent with the release of soluble CXCL16 by human prostate cancer cells reported recently (17). In contrast, accumulation of surface CXCL16 was shown in some human and mouse breast cancer cells, but release of the soluble form was not tested in this study (13). This difference could be due to differential expression of ADAM-10 and ADAM-17 by the breast cancer cells analyzed by Meijer *et al.* since they did not detect expression of these MPases in breast cancer (13). The cancer cells used in our study expressed both MPases (Fig. 5), consistent with the reported frequent expression of ADAM-10 and ADAM-17 in many tumors, including breast cancer (29,30).

Ionizing radiation at therapeutic doses has been shown to induce increased expression of several cell surface molecules on cancer cells [reviewed in ref. (26)]. Induction of some chemokines by ionizing radiation has also been reported. The chemokine IL-8/CXCL8 was induced by a dose of 6 Gy in human keratinocytes (31), and eotaxin/CCL11 was induced in human dermal fibroblast but not lung epithelial cells after a dose of 8 Gy (32). Extremely low-dose ionizing radiation (1 cGy) caused a transient up-regulation of three CXC chemokines (CXCL1, CXCL2 and CXCL6) in human fibroblasts (33). Therefore, other chemokines in addition to CXCL16 can be induced by ionizing radiation. However, the functional significance of this induction remains to be established. Up-regulation of gene expression does not always result in production of chemokines at levels likely to be physiologically significant, at least *in vitro* (Fig. 3 and Fig. 4). *In vivo*, it is possible that

local concentrations could reach levels sufficient to trigger some responses. Myeloid and endothelial cells present in the tumor stroma could also be sources of CXCL16 *in vivo*, but whether they respond to ionizing radiation by up-regulating CXCL16 remains to be determined.

In the case of CXCL16, we have shown that the radiation-induced levels of chemokine released by breast cancer cells were sufficient to drive a strong chemotactic response in activated T cells (12). The potential stimulation of cancer cell growth by soluble CXCL16 (13) may be negligible, since tumor cells receiving therapeutic doses of radiation are impaired, at least temporarily, in their proliferative ability. Therefore, there may be a window of opportunity after radiotherapy in which the enhanced propensity of a tumor to attract activated T cells can be exploited to achieve immune-mediated tumor rejection.

Other effects of radiation may contribute to enhance recruitment of T cells to tumors. Radiation has been reported to induce the expression of vascular cell adhesion molecule 1 (VCAM-1) on the vasculature of B16 melanoma, facilitating enhanced recruitment of activated T cells to irradiated tumors (34,35). Since B16 cells do not up-regulate CXCL16 (Fig. 3), VCAM-1 induction may play a critical role in this tumor. It is also likely that other chemokines capable of attracting T cells can be induced by radiation. Consistent with this hypothesis, Lugade *et al.* described the expression of CXCL9 and CXCL10 by B16 cells and its up-regulation by interferon γ (35), and we have results suggesting that there are common and distinct chemokines induced by radiation in different tumors (SM and SD, Fifty-fourth Annual Meeting of the Radiation Research Society, Boston, 2008).

Overall, the present data support the proposition that radiotherapy could be used to enhance the effectiveness of immunotherapy by facilitating the effector phase of the anti-tumor immune response. Additional studies are needed to determine the optimal use of radiation as an immunological adjuvant (36).

Acknowledgments

This work was supported by NIH R01 CA113851 and Research Scholar award RSG-05-145-01-LIB from the American Cancer Society to S. Demaria. We thank the personnel of NYU Cancer Institute Flow Cytometry facility for expert assistance.

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FIG. 1. CXCL16 mRNA expression in mouse cancer cell lines. Panel A: Expression of the CXCL16 (416 bp) and β-actin (188 bp) gene products. Panel B: Ratio of CXCL16 to β-actin calculated from densitometric quantification of the bands.

FIG. 2.

Expression of CXCL16 transmembrane form on cancer cells. Cells were incubated for 2 h with (solid line) or without (broken line) the MPase inhibitor BB-94 and stained with goat anti-CXCL16 antibody (solid and broken lines) or control medium (dotted line) followed by FITC-donkey anti-goat antibody. Numbers are the mean fluorescence intensity of cells incubated with (bold) or without (regular) BB-94 after subtraction of the background fluorescence of cells stained with control medium.

FIG. 3. Real-time RT-PCR measurements of the expression of CXCL16 in irradiated cancer cells. Data are the means $\pm$ SD of three independent experiments. Asterisks (*) indicate statistically significant difference ($P < 0.05$) compared to levels on untreated cells.

FIG. 4. Enhancement of soluble CXCL16 release by radiation. Data are the means of duplicate wells and are representative of two independent experiments. No detectable CXCL16 could be measured in the supernatants of B16/F10 and MC57 cells.

FIG. 5.

Expression of ADAM-10 (panel A) and ADAM-17 (panel B) in cancer cells determined by RT-PCR. The ratio of each MPase to β -actin was calculated from densitometric quantification of the bands.