Regulation of Interleukin-8 Expression in Human Melanoma Cells by the Organ Environment

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ABSTRACT

The in vitro expression level of interleukin-8 (IL-8) correlates with the metastatic potential of human melanoma cells. The purpose of this study was to determine whether the expression level of IL-8 in human melanoma cells is influenced by the organ microenvironment. A375P cells, a low metastatic human melanoma, and A375SM cells, a highly metastatic variant, were injected into the subcutis (s.c.), spleen (to produce liver metastases), and lateral tail vein (to produce lung metastases) of athymic nude mice. Northern blot and immunohistochemical analyses demonstrated that s.c. tumors, lung lesions, and liver lesions expressed high, intermediate, and low IL-8, mRNA, and protein, respectively. This differential regulation of IL-8 was not due to the size or density of the lesions or to selection of subpopulations of cells. We based this conclusion on the results of three experiments: (a) melanoma cell lines established in culture from in vivo-growing tumors exhibited similar levels of IL-8 mRNA transcripts; (b) in a crossover experiment, the level of IL-8 mRNA was always high in A375 tumors reestablished in the skin and low in the tumors reestablished in the liver, regardless of whether the melanoma cells had been first harvested from s.c. or liver tumors; and (c) A375 melanoma cells cocultured with human keratinocytes produced high levels of IL-8 protein, whereas A375 cells cocultured with highly differentiated human hepatoma cells produced decreased levels. When A375P cells were then incubated with cytokines associated with keratinocytes (IL-1 and interferon β) or hepatocytes (transforming growth factor α or β), IL-1 enhanced the production of IL-8 protein, whereas TGF-β decreased its production. These data show that IL-8 expression in melanoma cells is modulated by local host factors.

INTRODUCTION

Once metastatic cells reach specific organ parenchyma, they must proliferate to give rise to clinically detectable lesions (1). This growth depends on the interaction of the metastatic cells with the organ environment, which is itself mediated by autocrine and paracrine growth factors (1-3). Many homeostatic mechanisms can actually enhance the survival and growth of tumor cells in specific organs (1-5). Moreover, organ-specific cytokines or components of the extracellular matrix can influence the capacity of tumor cells to divide (3-5), produce angiogenic molecules (6), invade host stroma (7), and even respond to chemotherapeutic drugs (8).

Melanoma cells secrete a variety of growth factors either constitutively or subsequent to induction by other cytokines (9-11). These growth factors-cytokines may act as autocrine growth factors or modulate the host environment to stimulate growth (9-11). Melanoma cells treated with IL-1, which is released by keratinocytes (12, 13), express IL-8 (14). IL-8 is multifunctional and has been shown to induce proliferation of keratinocytes (15) and melanoma cells (15-17), induce angiogenesis (18, 19), and induce haptotactic migration in melanoma cells (20). Since all these phenotypes are important in the production of metastases, we examined whether IL-8 expression correlated with the metastatic potential of human melanoma cells. Indeed, we found a direct correlation between the constitutive expression of IL-8 in cultures of human melanoma cells and their ability to produce metastasis in nude mice (17). Since IL-8 expression in melanoma cells can be induced by cytokines such as IL-1 and tumor necrosis factor α, the question of whether specific organ environments could influence expression of IL-8 in melanoma cells remained unanswered.

The purpose of this study was to examine whether the expression of IL-8 in melanoma cells can be influenced by a specific organ environment. We show that melanoma cells growing in the skin express high levels of IL-8, whereas the same cells growing in the liver do not. The differential expression of IL-8 was due to adaptation to the local organ environment and regulation by different cytokines expressed in the specific environment.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The low metastatic A375P human melanoma cell line was originally established in culture from a lymph node metastasis of a 54-year-old female melanoma patient (21). The A375SM metastatic line was established in culture from lung metastases produced by the A375P cells growing s.c. in nude mice (21). Neonatal human keratinocytes were purchased from Clonetics Corporation (San Diego, CA). C3A is a human hepatoma cell line that retains most of the properties of normal hepatocytes, including biochemical function and growth characteristics (22). The C3A cells are being used in bioreactors as a bioartificial liver (22) and were the gift of Dr. J. Kelly (Hepatix, Inc., Houston, TX).

The cell lines were maintained in culture as adherent monolayers in Eagle’s MEM supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, 1-glutamine, 2-fold vitamin solution, and penicillin-streptomycin (Flow Laboratories, Rockville, MD). Human keratinocytes were grown in serum-free keratinocyte growth medium (Clonetics) supplemented with bovine pituitary extract. C3A cells were maintained in Hepatix MM medium (Hyclone Laboratories, Inc., Logan, UT). All cultures were free of Mycoplasma and pathogenic murine viruses (assayed by Microbiological Associates, Bethesda, MD). Cultures were maintained for no longer than 4 weeks after recovery from frozen stocks.

Animals. Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

Tumor Cell Injections. To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by a brief exposure to a 0.25% trypsin-0.02% EDTA solution (w/v). The flask was sharply tapped to dislodge the cells, and supplemented medium was added. The cell suspension was pipetted to produce a single-cell suspension. The cells were washed and resuspended in Ca2+- and Mg2+-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of greater than 90% viability were used.

s.c. tumors were produced by injecting 1 x 10^6 tumor cells/0.2 ml HBSS over the right scapular region. Experimental lung metastases were produced by...
IL-8 Production by A375 Melanoma Cells Cocultured with Human Keratinocytes or Human Hepatoma Cells. Human keratinocytes and C3A hepatoma cells were seeded at a density of 1 × 10^5 cells/38-mm² well of 96-well plates and incubated to near confluence, at which time 1 × 10^5 A375P or A375SM cells were added. Tumor cells, keratinocytes, or C3A cells cultured alone served as controls. Supernatants of triplicate cultures were examined in a bright-field microscope. A positive reaction was indicated by a reddish brown precipitate in the cytoplasm.

IL-8 Production by A375 Melanoma Cells Treated with Cytokine-Growth Factors. We plated 5 × 10^5 A375P or A375SM cells into 38-mm² wells of 96-well plates. After 24 h, the cells were washed with serum-free MEM and refed with supplemented MEM containing IL-1α (Boehringer-Mannheim, Indianapolis, IN), IFN-α, IFN-β (Biosource International, Camarillo, CA), or TGF-α (Oncogene Science, Uniondale, NY), and TGF-β (R&D Systems, Minneapolis, MN). Cell-free culture supernatants were collected at different time points and analyzed for IL-8 protein by an ELISA.

RESULTS

IL-8 Expression in A375 Melanoma Tumors from Skin, Lung, and Liver of Nude Mice. In the first set of experiments, we analyzed IL-8 mRNA in A375P and A375SM cells growing in culture or in different organ sites of nude mice. Low metastatic A375P cells expressed low levels of IL-8 transcripts in culture (Fig. 1, Lane A). Compared with the cultured cells, IL-8 expression by A375P cells increased 7-fold in s.c. lesions, doubled in lung metastases (Fig. 1, Lane B), and decreased in liver lesions (Fig. 1, Lane C). Highly metastatic A375SM cells expressed high levels of IL-8 transcripts in culture (Fig. 1, Lane E). Compared with the cultured cells, IL-8 expression by A375SM cells was slightly increased in the s.c. tumors, low in the liver metastases, and identical in the lung metastases (Fig. 1, Lanes F-H). Immunohistochemical staining of the A375P melanoma tumors showed an intense positive staining in cells growing s.c. (Fig. 2A) but not in cells in liver lesions (Fig. 2).

We next determined whether the expression of IL-8 in A375P melanoma growing in vivo was associated with the size of the lesions. Mice were injected s.c. with 5 × 10^5 A375P cells. Small (3 mm in diameter) and large (10 mm in diameter) tumors were harvested. Large tumors were also produced by injecting 2 × 10^6 cells s.c. and

Fig. 1. Northern blot analysis from A375P and A375SM cells in culture and as tumors in the s.c., liver, and lungs of nude mice. Poly(A)+ mRNA (2.5 μg/lane) was probed with a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8, from which 1.8-kb transcript is expected (25), and a rat glyceroldehyde-3-phosphate dehydrogenase (GAPDH) cDNA that detects a human 1.3-kb transcript (24). The absorbance of each IL-8 band was calculated as the ratio between the areas of the IL-8 and GAPDH transcripts. The IL-8/GAPDH ratio of cultured cells was designated as 1.0. Data are from one of three representative experiments.
ORGAN ENVIRONMENT-REGULATED IL-8 IN MELANOMA CELLS

Fig. 2. Immunohistochemical staining of A375P melanoma tumors with anti-IL-8 antibodies. A, A375P cells growing as a s.c. tumor showing intense histochemical reaction (× 200). B, A375P melanoma growing as liver metastases subsequent to intrasplenic injection (× 200). There is no staining of tumor cells; a rim of compressed liver cells, staining slightly positive with anti-IL-8 antibody, can be seen.

Harvesting tumors after 2 weeks. mRNA was extracted from both small and large s.c. tumors. Northern blot analysis for the expression of IL-8 mRNA transcripts failed to reveal differences in IL-8 expression between small and large tumors (Fig. 3). The same was true when we compared IL-8 expression in sparse and confluent cultures of A375P (Fig. 3A).

The Differential Expression of IL-8 in the Skin and Liver Is Due to Adaptation to the Organ Environment. In the next set of experiments, we determined whether the level of IL-8 mRNA expression in the s.c. and liver tumors was due to selection of a subpopulation of cells or to adaptation to the organ environment. Nude mice (n = 5) were given injections of 5 × 10⁵ A375P cells in the subcutis, spleen, or lateral tail vein. Melanoma lesions were harvested aseptically. One aliquot was frozen in liquid nitrogen for mRNA extraction, and the other was mechanically dissociated and its tumor cells established in culture. After 7 or 14 days of culture, RNA was extracted from the cells. IL-8 mRNA expression in the tumors and the cell lines was analyzed by Northern blot. A375P cells growing s.c. expressed 3-fold the level of IL-8 mRNA transcripts as compared with cells growing in continuous culture (Fig. 4, compare Lane A with Lane B). The high expression of IL-8 mRNA transcripts was transient. After 2 weeks in culture, A375 cells established from s.c. lesions expressed the same (low) levels of IL-8 mRNA as that found in cells growing in vivo and adapted to culture. Nude mice were given injections of 5 × 10⁵ A375P cells into the s.c., spleen, or lateral tail vein. S.c., liver, and lung lesions were harvested aseptically, and cells were established in culture. mRNA was extracted from tumor tissue, and cells were cultured for 1 and 2 weeks. The IL-8:GAPDH ratio of each sample was compared to A375P cells growing in culture (Lane A).
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Fig. 5. Expression of IL-8 mRNA in A375P cells growing in the s.c. or liver of nude mice (cross-over study). A375P cells were injected s.c. and into the spleen of nude mice to produce liver lesions. Tumor cells from the s.c. and liver lesions were adapted to culture and injected under the skin (Lanes B and D) and into the spleen (Lanes C and E) of nude mice to produce liver lesions. mRNA was extracted from the resulting tumors, and 2.5 µg poly(A)+ mRNA was analyzed by Northern blot. The absorbance of each sample was compared to the IL-8:GAPDH ratio in A375P cells growing in culture (Lane A).

Fig. 6. Production of IL-8 protein by A375 cells cocultured with human keratinocytes or C3A hepatoma cells. A375P or A375SM melanoma cells were added to confluent cultures of keratinocytes (A and B) or C3A hepatoma cells (C and D). Culture supernatants were harvested 24, 48, 72, and 96 h later, and IL-8 secretion was determined by ELISA and expressed as pg/ml. Coculture of A375P (A) or A375SM (B) cells with keratinocytes resulted in up to a 3-fold increase in IL-8, whereas coculture of A375P (C) or A375SM (D) cells with C3A hepatocytes resulted in a reduction of IL-8 production. This is one representative experiment of two.

continuous culture (Fig. 4, Lanes C and D). The IL-8 expression in A375P cells established in culture from liver lesions was initially low, but within 1 week, it returned to that found in continuous cultures (Fig. 4, Lanes E, F, and G). The mRNA expression of IL-8 in lung metastases doubled as compared to parental cells growing in culture but likewise returned to baseline level after 2 weeks in culture (Fig. 4, Lanes H–J).

To further examine whether the level of IL-8 expression in A375P cells was due to adaptation to the organ environment, we carried out a cross-over experiment. Cultures of A375P cells were established from s.c. or liver tumors, and these cells were injected into the s.c. or spleen of nude mice. Tumor lesions from the s.c. and liver were harvested for mRNA extraction, and IL-8 transcripts were analyzed by Northern blot. Regardless of the origin of the cells, the expression of IL-8 mRNA transcripts was high in s.c. melanoma and low in the liver melanoma lesions (Fig. 5).

IL-8 Production by A375 Melanoma Cells Growing in Coculture with Human Keratinocytes or C3A Hepatoma Cells. In the next set of experiments, we cocultured A375P cells with either human keratinocytes (skin environment) or highly differentiated C3A hepatoma cells (liver environment). Culture supernatants were harvested after 24, 48, 72, or 96 h. A375P and A375SM cells cocultured alone were used as controls. IL-8 levels in the keratinocytes and C3A hepatoma cells were low, measuring 109 ± 12 and 92 ± 12 pg/ml/10⁶ cells in 48-h cultures. A375P cells cocultured with keratinocytes produced an increased level of IL-8 (Fig. 6A). A375SM cells produced less of an increase (Fig. 6B). A375P or A375SM cells cocultured with C3A hepatoma cells produced a decreased level of IL-8 as compared with melanoma cells cultured alone (Fig. 6, C and D). The coculture experiments were carried out twice with similar results.

Regulation of IL-8 Expression by IL-1 and TGF-β. In the last set of experiments, we identified cytokines that could replicate the respective stimulation and inhibition of IL-8 expression in A375 cells cocultured with keratinocytes or hepatoma cells. Among several cytokines, keratinocytes produce IL-1, IFN-β, and to a lesser extent, IFN-α (26, 27), whereas hepatocytes produce TGF-α and TGF-β (28). For this reason, 1 X 10⁶ A375P cells were seeded into culture wells, and 24 h after plating, IL-1 (10 units/ml), IFN-α (10 units/ml), IFN-β (10 µg/ml), TGF-α (10 ng/ml), or TGF-β (10 ng/ml) were added. One day later, cell-free supernatants were analyzed for IL-8 protein by ELISA. IL-8 protein production tripled in A375P cells incubated with IL-1 (Fig. 7A). Incubation of A375P cells with TGF-β produced 50%

Fig. 5. Expression of IL-8 mRNA in A375P cells growing in the s.c. or liver of nude mice (cross-over study). A375P cells were injected s.c. and into the spleen of nude mice to produce liver lesions. Tumor cells from the s.c. and liver lesions were adapted to culture and injected under the skin (Lanes B and D) and into the spleen (Lanes C and E) of nude mice to produce liver lesions. mRNA was extracted from the resulting tumors, and 2.5 µg poly(A)+ mRNA was analyzed by Northern blot. The absorbance of each sample was compared to the IL-8:GAPDH ratio in A375P cells growing in culture (Lane A).
inhibition in IL-8 protein production (Fig. 7B). IFN-α, IFN-β, and TGF-α did not affect the level of IL-8 protein in the cells. The experiments were repeated twice with similar results.

DISCUSSION

The influence of the organ environment on the growth of tumor cells was originally proposed in Paget’s hypothesis of “the seed and the soil” (29). More recently, experimental data have shown that the organ environment influences tumorigenesis (2); the production of degradative enzymes (7, 30), melanin (31, 32), and angiogenic molecules (6); the induction of terminal differentiation (33); and the level of P-glycoprotein associated with the multiple drug resistance phenotype (8, 34). Since IL-8 has been shown to enhance the growth of malignant melanoma (12, 16, 17), we determined if its expression is also influenced by host factors associated with specific organs.

Human A375 cells implanted into the s.c. of nude mice or growing as experimental lung or liver metastases expressed high, intermediate, and low levels of IL-8, respectively. The differences in expression of IL-8 were not due to selection of different subpopulations of cells but rather to adaptation to the organ environment; A375 cells established in culture from s.c., lung, or liver lesions produced the same level of IL-8 (after 7–14 days of culture), and in a cross-over experiment, tumors first established in the skin or liver exhibited high levels of IL-8 when reestablished in the skin and low levels when reestablished in the liver, regardless of the origin of the implanted cells.

To determine whether local host cells could account for the modifications in IL-8 expression, we cocultured melanoma cells with two cell lines representing “normal host cells.” Human keratinocytes were used to imitate the skin microenvironment. Keratinocytes are the predominant cell type in the epidermis (14). They were found to regulate melanocyte number and position during epidermal development (35). Keratinocytes have also been the target of experimental manipulations, such as UV irradiation, performed to determine their role in melanoma progression (36). In our study, coculture of A375 melanoma cells with keratinocytes led to a significant increase in IL-8 concentrations in the supernatants, suggesting that keratinocytes in the nude mouse skin up-regulate IL-8 production. Furthermore, when A375 cells were incubated in the presence of IL-1, IFN-α, and IFN-β, all known to be produced by keratinocytes (11–13, 26, 27), IL-1 was associated with a 3-fold increase in IL-8 production by A375 melanoma cells. Since neither A375 nor A375SM cells produce IL-1 (37), these data suggest that IL-1 released by normal host cells influences IL-8 production in the melanoma cells. The coculture data, however, do not rule out that under in vivo conditions, additional cytokines can influence IL-8 expression in melanoma cells.

Since normal hepatocytes do not divide in culture, we cocultured A375 cells with cells of the highly differentiated, C3A hepatoma that produces IL-1, IFN-α, and IFN-β, all known to be produced by keratinocytes (12, 13), can induce IL-8 production in tumor cells (47) and support the hypothesis that angiogenesis surrounding s.c. melanoma lesions could be due to overexpression of IL-8. The abrogation of IL-8 expression in melanoma cells growing in the liver suggests that this cytokine is not necessary for continuous growth of melanoma cells in this organ. The mechanism by which IL-1 and TGF-β regulate IL-8 production by human melanoma cells is now under active investigation with a view to future therapeutic intervention.

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