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MODIFICATION OF TUMOR MICROENVIRONMENT BY CYTOKINE GENE TRANSFER

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The tumor microenvironment is determined by the interactions between host and tumor cells, a process in which cytokines play a major role. We have used retroviral vectors to insert and express cytokine genes in tumor cells so as to induce predictable changes in the host cells that infiltrate tumors. This frequently caused changes in tumor cell phenotype through autocrine/intracrine pathways. We reasoned that cytokine-induced alterations in tumor cell phenotype and/or in infiltrating host cells might alter the in vitro and in vivo cellular response to irradiation. In the present paper we document some of the effects of expression of interleukin-6 (IL-6) and IL-7 genes in tumor cells in this regard. The studies support the hypothesis that cytokines may play a role in determining both intrinsic tumor radioresponsiveness and the tumor microenvironment and in these ways may influence in vivo tumor irradiation responses. Possible cytokine gene-mediated approaches to radiotherapy cancer are discussed.

The microenvironment within a solid tumor mass is not solely defined by the properties of the malignant cell population but rather is a product of the interaction between tumor and host-derived cells. Manipulation of either the type or the functional activity of host cells in tumors is therefore likely to modify the tumor microenvironment. One way of achieving this is by the use of cytokines, although much has yet to be learned as to how specific cytokines might alter the tumor microenvironment and how this might influence the response to chemo-, radio-, or immunotherapy.

We (1–4), and others (5–12), have studied in vitro and in vivo the effects of genetic manipulation of tumor cells to release large amounts of a chosen cytokine over an extended time period. We recently reviewed the results of our own studies and of the literature in this field (1, 2). It is clear from these studies that specific cytokines can drive consistent changes in local microenvironments and that this is a more effective, less toxic, and more physiological form of cytokine delivery than the systemic route because of the formation of concentration gradients.

The results of preclinical trials with experimental and human tumors genetically manipulated to produce proinflammatory or immune cytokines have led to the design of numerous clinical protocols for cancer using genetically altered tumor cell vaccines. The basis for these protocols is the repeated observation that expression of practically any pro-inflammatory cytokine decreases tumorigenicity in vivo by stimulating host cell anti-tumor defenses. In addition, certain cytokines can enhance tumor immunogenicity. In the longer term, delivery of cytokine genes directly into tumors would seem a promising approach to cancer therapy, but the addition of radiotherapy, chemotherapy, or surgery may be required for full effectiveness and there is a need to understand the interactions between these modalities.

It is pertinent to note that, in addition to stimulating a host cell response, manipulation of cytokine gene expres-

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sion by tumor cells often alters their in vitro phenotype through autocrine or intracrine action. Alterations in radiotherapeutic responses under conditions defined by cytokine secretion therefore might result from either changes in intrinsic radioresponsiveness, or in host cell content and function, or both. We have studied the impact of expression of certain cytokine genes on tumor cell radiation response in vitro and in vivo. Our results support the view that endogenous cytokine secretion can modify the intrinsic radioresponsiveness of tumors measured in vitro and in vivo. They also indicate a need for preclinical studies to evaluate the role of cytokines in radiation responses of tumors and of cytokine-based approaches to genetic radiotherapy of cancer.

Material and Methods

Tumors and tumor assays. The FSA tumor cell line was derived from a methylcholanthrene-induced tumor of moderate immunogenicity originating in C3Hf/Kam//Sed mice (3). The human osteosarcoma MG63 cell line was obtained from the American Type Culture Collection. In vitro irradiation was done using a Gammacell 220 (Atomic Energy Limited, Canada) with a cobalt source and at a dose rate of 3.3 Gy/min. After irradiation cells were plated in 100 mm Petri dishes and colonies of greater than 50 cells counted on day 12 after staining by Giemsa (13). For in vivo tumor irradiation, 5×10^6 FSA parental or gene transduced cells were injected s.c. in the thigh of C3Hf/Kam//Sed mice and tumors irradiated when 6 mm in diameter using a 250 kVp Phillip's x-ray source (dose rate 1.2 Gy/min) with the rest of the body shielded. The high cell number was needed to overcome the decreased tumorigenicity of the cytokine gene-transduced cells (4). Flow cytometric analysis of cell cycle arrest was performed as described previously (13). The data presented are representative of 3 separate experiments and, where shown, error bars signify mean $\pm$ 1 S.E.M.

Gene transduction. The murine myeloproliferative retrovirus-based vector, Jzen.1 described previously (3, 4), was used in these studies. Full length hIL-6 or mIL-7 cDNA inserts were driven off the 5'LTR and all constructs contained a neomycin resistance gene driven by an internal thymidine kinase (Tk) promoter. The mIL-7 vector was transfected into the ecotropic packaging cell line GP + env86 and the hIL-6 vector into amphotropic GP + envAM12 cells. Cloned cell lines were derived which produced virus titers in excess of 10^6 infectious viral particles/ml. Tumor cell lines were infected with Jzen cytokine-Tkneo or control Jzen-Tkneo (Jneo) viral supernatants and infected cells were selected in G418. The efficiency of gene transfer was in the range 25–50%. Northern analyses confirmed that cytokine gene-transduced cells expressed the appropriate proviral mRNA, and ELISA and biological assays were used to measure levels of cytokine production and to confirm biological activity. Levels of secreted cytokine were in both cases in the range 20–30 ng/ml/10^6 cells. Parental cells or cells infected with Jzen Tkneo (FSA-Jneo or MG63-Jneo) did not produce IL-7 or IL-6.

Results

In vitro radiation responses and cytokine gene expression. Cytokine gene insertion and expression can act through feedback loops to alter tumor cell phenotype (see (1, 2)). We considered the possibility that such loops might alter the response of tumors to therapeutic agents such as radiation. If this were to be the case cytokine gene transfer might be an approach to modifying tumor radioresistance. Transduction of the human osteosarcoma cell line MG63 with the gene for the proinflammatory cytokine IL-6 was found to significantly increase in vitro sensitivity of this cell line to irradiation as judged by colony formation (Fig. 1). IL-6 expression slowed proliferation of this cell line from a doubling time of 16.3 h to 31.2 h and the proportion of MG63-JhIL-6 cells arrested in G_2M phase after 2 Gy irradiation was increased over control (Fig. 2). Insertion of the control neo vector did not affect proliferation or radiosensitivity of the cell line.

Transduction of the murine FSA cell line with the gene for IL-7, which plays a role in T and B lymphocyte development and proliferation (see (1)), did not obviously affect its proliferation (4), although the growth of several human tumor cell lines was slower after hIL-7 gene transduction (Dubinett & McBride, unpublished study). The phenotype of the FSA cells is, however, changed by IL-7 gene expression. FSA-JmIL-7 cells grown in vitro had decreased levels of the immunosuppressive cytokine TGF-β (14), indicating that an autocrine/intracrine pathway was active. That this is a property of IL-7 is also indicated in the observation that exogenous IL-7 down-regulates TGF-β production by macrophages (15). In vitro colony form-

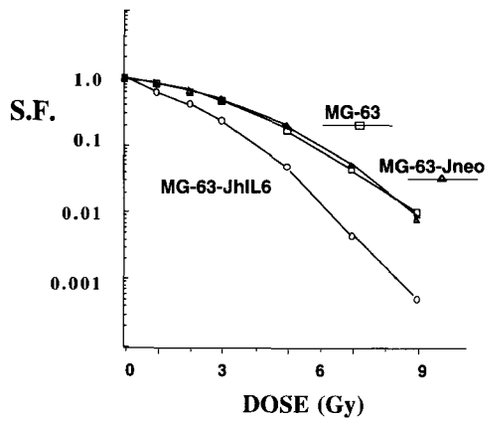

Fig. 1. The in vitro response of the human osteosarcoma cell lines MG63-JhIL-6, MG63-Jneo, and MG63 to ionizing radiation as measured by a clonogenic assay.

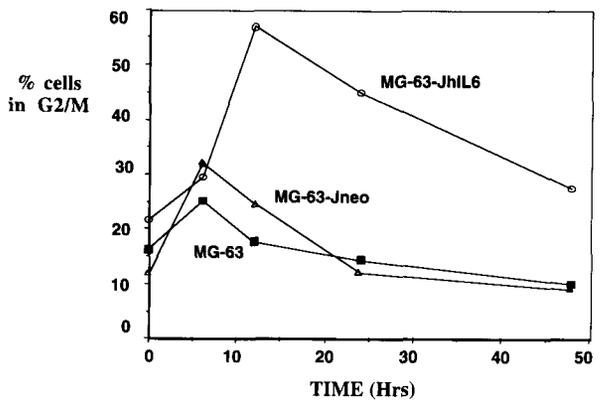

Fig. 2. The in vitro response of the human osteosarcoma cell lines MG63-JhIL-6, MG63-Jneo, and MG63 to ionizing radiation as measured by the % of cells in G_2M phase of the cell cycle at varying times after irradiation. Analysis was performed by flow cytometry after staining with propidium iodide.

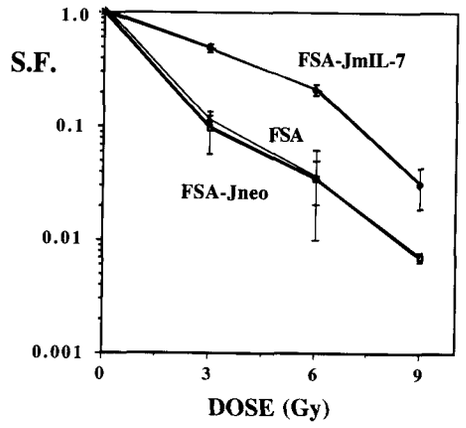

Fig. 3. The in vitro response of murine fibrosarcoma cell lines FSA-JmIL-7, FSA-Jneo, and FSA to ionizing radiation as measured by a clonogenic assay.

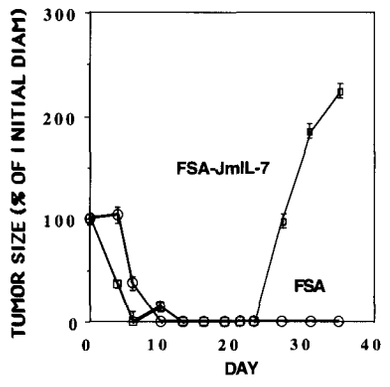

Fig. 4. The in vivo response of murine fibrosarcoma cell lines FSA-JmIL-7, FSA-Jneo, and FSA to ionizing radiation as measured by regrowth after 50 Gy irradiation. Tumors growing in the thigh were irradiated when 6 mm in diameter.

ing assays showed that FSA-JmIL-7 was more radiation resistant than the FSA-Jneo or FSA controls (Fig. 3).

In vivo radiation responses and cytokine gene expression. Inherent in the reasoning that the tumor microenvironment is determined by the tumor-host interaction is the concept that the response to therapy will depend not only on tumor cell properties but also on the nature of the host cell response. We have previously reported (4) that FSA-JmIL-7 cells have decreased tumorigenicity and increased immunogenicity in vivo. Significantly, 25% of the FSA-JmIL-7 tumors that grew in excess in 5 mm in diameter were spontaneously rejected by a T cell-dependent mechanism (4), consistent with the known ability of this cytokine to stimulate T cells. Mice that rejected tumor became immune to rechallenge with the same, but not an antigenically unrelated tumor. By flow cytometry, FSA-JmIL-7 tumors growing in mice had greatly increased T cell numbers, some eosinophils and some basophils (4). Control FSA or FSA-Jneo tumors contained on average 55% tumor cells, the rest being host cells. In the case of FSA-JmIL-7, T cells made up 30-50% of the tumor and tumor cells 20–40%, the proportion presumably reflecting the extent of tumor regression. In spite of the strong T cell-mediated host cell response, in a series of preliminary studies 6–8 mm diameter FSA-JmIL-7 tumors were more resistant to high single dose irradiation than controls (Fig. 4).

Discussion

The determinants of tumor resistancy are complex and it would be misleading to draw too many conclusions from these initial studies. The mechanism by which intrinsic radiation responsiveness is altered by cytokine expression has yet to be elucidated, but, signal transduction pathways that determine radioresistance or radiosensitivity are beginning to be defined largely by study of the effects of oncogene expression (16), and specific cytokines could drive similar pathways. Altered cell proliferation, cell adhesion, or cell differentiation are likely to impact the radiation response outcome and the effect of the cytokine in these regards will depend at least in some situations on the genetic program of the cell. Thus far in our studies, cytokines that slowed cell proliferation most often sensitized cells to irradiation in vitro and vice-versa, although it would be premature to generalize. Increases in the number of cells arresting in G2 phase after irradiation have been found for radiation-sensitive ataxia cells (13), although the opposite relationship has been found for certain oncogene-transfected cell lines (16). The rate of cell cycle progression seems to be important in determining the extent of G2 arrest (Chiang & McBride, unpublished study), but it seems likely that it also depends on other properties, such as adhesion related molecules. It should be noted in these and other studies that the long-term effects of cytokine gene expression on tumor phenotype, including radiation response, has yet to be examined in detail and, although

such changes are assumed to be permanent as long as the gene is expressed, more subtle long-term alterations may take place that could influence the outcome. An important implication from our findings is that endogenous cytokine expression may play a role in determining intrinsic cellular radiosensitivity and may serve in some situations as a predictive marker of radiation response.

The in vivo situation is more complex than in vitro. The impact of the tumor microenvironment has to be taken into account as well as intrinsic cellular radiation sensitivity when considering mechanisms of radioresponsiveness. In spite of the enhanced T cell infiltrate, FSA-JmIL-7 tumors were more radioresistant in vivo. Further studies are needed to examine the mechanism responsible for this effect. It could be due to increased intrinsic radioresistance but the data suggest that a radioresistant subpopulation, perhaps a hypoxic one, may be responsible. Cytokine expression is likely to determine the level of vascularization and hypoxia in tumors and this, in addition to the host cell content, could be altered in FSA-JmIL-7 tumors. Whatever the mechanism, the findings raise interesting questions as to the possible influence of different components of the immune system on tumor radioresponsiveness. A relationship between macrophage content and radioresistancy has been reported previously (16), and it may be more than coincidence that some of the more radioresistant tumors such as melanoma, renal cell, and lung carcinoma, are more immunogenic and heavily host cell-infiltrated. Overall the studies support the hypothesis that under normal conditions host and tumor cell interactions are intimate and extensive—not only is the host cell infiltrate defined by the tumor but there is a two-way interaction in which host cells may influence tumor cell phenotype and these may influence the response to therapy.

These studies point to an important role for cytokines in determining tumor responses to therapy, acting both through autocrine/intracrine pathways and/or through host cells. They suggest that manipulation of tumor responses to chemo- or radiotherapy might be possible by cytokine gene transfer, although many more studies are needed to determine the predictability and reproducibility of these effects. The genetic programs of the tumor and the host will play a role in determining the nature of the response and the impact of cytokines will have to be carefully studied in different tumor types. In addition, if gene therapy is to be successful, the problem of gene delivery to tumor has to be overcome. Most current experimental studies and clinical protocols use retroviral vectors to introduce and express cytokine genes in tumor cells. The major advantages of retroviral vectors are the high efficiency of gene transduction and gene integration into the genome which can allow prolonged levels of expression, typically in the range 1–50 ng cytokine/ml/10^6 cells for many months. The major disadvantages are that cells have to be proliferating to be infected with retrovirus and that in vivo transduction frequencies are low. Adenoviral vectors have advantages of high infectivity for proliferating and non-proliferating cells alike, both in vitro and in vivo, though they are episomal vectors that do not integrate into the genome so their persistence is likely to be for a shorter time period. The advantages and disadvantages of these and other vectors have yet to be fully established, as are methods for specifically targeting genes to tumors and on these will depend the efficacy of gene radiotherapy.

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