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Intradermal Injection, as Opposed to Subcutaneous Injection, Enhances Immunogenicity and Suppresses Tumorigenicity of Tumor Cells[1]

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ABSTRACT

Tumor cell immunogenicity depends heavily upon the microenvironment in which the cells grow. We have compared the tumorigenicity and immunogenicity of the same tumor cells when injected either into the dermis, a tissue containing numerous dendritic cells (DCs), or s.c., at a site which contains only few DCs. After s.c. injection, progressive tumors were constantly obtained, whereas most intradermal injections did not give rise to tumor and immunized animals against additional challenge. We present evidence that the high density of DCs at dermal sites facilitates the capture of tumor antigens and that local inflammation induces maturation of the DCs and their migration into draining lymph nodes.

INTRODUCTION

Several studies have demonstrated the trafficking of DCs[3] from the site of antigen capture to the draining lymphoid organs (1–3). Immature DCs such as Langherans cells capture tumor antigens and, under the influence of inflammatory stimuli, subsequently migrate to T-cell-rich areas such as lymph nodes where they can present antigen to naïve T cells and generate a tumor-specific immune response (2). The density of infiltrating DCs, the amount and form (4) of tumor antigen release, and the activation of these DCs by danger signals seem to be the most critical features for inducing an antitumor immune response (2). Using the experimental system PRO/REG (5), we previously demonstrated that tumor antigen release after s.c. injection is an important feature to induce an antitumor immune response (6).

In this study, we compared the tumorigenicity and immunogenicity of tumor cells injected either into the dermis, a tissue containing numerous DCs, or s.c., at a site which contains only few DCs. Most experimental rodent tumor models use s.c. injection of tumor cells to yield tumors in rodents. s.c. tissue is not rich in DCs, and s.c. injection does not induce local inflammation. In contrast to s.c. tissue, the skin is very rich in DCs such as Langerhans cells, which infiltrate the squamous epithelial cells of the epidermis and dermal DCs (3, 7, 8). Intradermal (i.d.) injection of tumor cells is not routinely practiced in the rodents, mainly because the thinness of dermis makes rigorous i.d. injection difficult and uncertain. We used PRO tumor cells, which after s.c. injection in rats, give rise to PRO, metastatic, and lethal tumors. PRO cells induce also peritoneal carcinomatosis when given i.p., multiple pulmonary metastases when administrated i.v., and liver nodules when injected into the spleen or the portal vein. s.c. PRO tumors induce immune tolerance because they enhance tumorigenicity and progression of highly immunogenic clones from the same tumor, the REG clones, which normally yield tumors that regress spontaneously in naïve rats (9).

In the present work, we demonstrated that i.d. administration of PRO tumor cells did not yield tumor in most injected animals. This tumor rejection is mediated by T cells as i.d. PRO tumor cells injected in nude rats always gave PRO tumors. Furthermore, all of the immunocompetent rats that have rejected the tumor cells after i.d. injection were vaccinated against additional s.c. tumor cell challenge. Immunohistological analyses performed on tumor injection sites showed a denser infiltration of DCs after i.d. tumor cell injection compared with s.c. injection. Furthermore, after i.d. but not s.c. injection of FITC-labeled tumor cells, DCs that have engulfed fluorescent tumor cell fragments could be recovered from the draining lymph nodes. Analysis of thymidine radioactive incorporation and IFN-γ production showed the presence of activated T cells in draining lymph nodes after i.d. but not after s.c. tumor cell injection. We also found that i.d. but not s.c. tumor cell injection induced local transcription of TNF-α, a major cytokine of inflammation that induces maturation of immature DCs into antigen-presenting cells. Thus, the high density of DCs and the local inflammation induced by the i.d. injection facilitate the capture of tumor antigen by immature DCs and their maturation into mature DCs and could be responsible for the subsequent rejection of a tumor cell line. These data show that the route of vaccine administration, as well as the site of the tumor growth, can have profound effects on outcome and may explain some of the variability that is often observed in *in vivo* vaccine experiments.

MATERIALS AND METHODS

Animals/Cell Lines/Tumorigenicity Assays. Nude rats and BD-IX rats were purchased from Charles River's Company (Wilmington, MA). In all of the animal experiments, we have observed the "Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education" issued by the New York Academy of Sciences. The progressive variant cell line (PRO), established from a rat colon adenocarcinoma, was cultured as described previously (5). For the tumorigenicity assays, 1×10^6 PRO tumor cells in 100 μl of serum-free medium were injected s.c. or i.d. into the thoracic wall or i.d. in the ear. Rats were re-challenged by s.c. injection of 1×10^6 PRO tumor cells in the flank. Tumor volume was evaluated weekly, using a caliper to measure two diameters.

Histological Study of the Tumor Cell Injection Site. Animals were sacrificed 4 or 7 days after tumor cell injection. An immunohistochemical study was performed as described previously (1). Mouse mAb to rat immature DCs and monocytes (ED1), mature macrophages (ED2), MHC class II (OX-17), CD3 (R7/3), CD4 (W3/25) and CD8 (OX8), and IgG isotype control were obtained from Serotec (Oxford, United Kingdom).

Fluorescent Labeling of Tumor Cells. As previously described (1), 10×10^6 PRO cells were incubated for 30 min at 37°C with FITC (Fluka, Buchs, Switzerland) at a concentration of 500 μg/ml and washed until no fluorescence was detected in the supernatant.

Isolation of Lymph Nodes Cells. Rats were killed 4 days after i.d. or s.c. tumor cell injections. Draining lymph nodes were taken out, pooled for each experimental group, and mechanically disaggregated through a steel wire mesh. After washing, lymph nodes cells were suspended in RPMI 1640 supplemented with 1% sodium pyruvate, 50 μM 2-mercaptoethanol, and 10% FCS (Life Technologies, Inc., Rockville, MD).

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[3] The abbreviations used are: DC, dendritic cell; PRO, progressive; REG, regress; TNF-α, tumor necrosis factor α; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ags, antigens.

Proliferation Assay and IFN-γ Measurement. T cells recovered from the draining lymph nodes 4 days after i.d. or s.c. PRO tumor cell injection were suspended (10^6 cells/ml) in a round-bottomed 96-well plate (Nunclon, Roskilde, Denmark). They were cocultured in triplicate with mitomycin-preincubated PRO tumor cells (1×10^5 cells/ml). After 3 days of coculture, T-cell proliferation was measured as previously described (1), and IFN-γ concentrations were measured in the supernatant using ELISA kit for rat-IFN-γ (Quantikine; R&D, Oxon, United Kingdom).

Cytofluorimetry Analysis. Cells were washed in PBS supplemented with 0.5% BSA and 0.01% sodium azide, adjusted to 1×10^5 cells/100 μl, and analyzed with a FACScan. The CellQuest software was used to determine the percentage of fluorescent cells after the engulfment of FITC-labeled proteins and the number of MHC class II-positive cells.

Reverse Transcription-PCR Analysis. A piece of cutaneous tissue ($1 \times 1 \times 0.5$ cm) was excised 6, 24, and 48 h after i.d. or s.c. injection in 3 rats in each group. Total RNA was extracted (RNeasy Mini Kit; Qiagen, Inc., Valencia, CA) and pooled in each group. A total of 2.5 μg of total RNA was used in first strand cDNA synthesis, priming with oligo$(dT)_{15}$ (Boeringer Mannheim). PCR cycling conditions were 94°C for 10 min, 94°C for 1 min, 54°C for 45 s, and 72°C for 1 min, with steps 2–4 repeated 30 times. Primer sequences were as follows: *TNF-α*, 5′-GCACCATGAGCACGGAAAGC-3′ and 5′-GCTCACAGAGCAATGAC-3′; and *GAPDH*, 5′-CTGGTGCTGAGTATGTCGTG-3′ and 5′-CAGTCTTCTGAGTGGCAGTG-3′. Semiquantitative PCR amplifications were performed with coamplifying GAPDH (294 pb) and TNF-α (750 pb). PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

RESULTS

Different DC Density in i.d. and s.c. Tissues. One million PRO tumor cells were injected s.c. or i.d. in naive syngeneic hosts. Only i.d. injection induced a swelling of the dermis and was followed by a moderate local inflammation during the first 2 days. To validate our observation and methodology, histological analyses were also performed just after i.d. or s.c. injection of tumor cells and were used to demonstrate the skin level (epidermidis, dermis, or hypodermis) in which the tumor cells were actually deposited. i.d. tumor cells (*) injection leads to a swelling of the dermis (Fig. 1*A*), whereas s.c. injections introduce tumor cells (*) under the hypodermis (Fig. 1*B*). Immunohistological analysis confirmed that 48 h after i.d. injection, the tumor cells were densely infiltrated by numerous ED-1-positive immature DCs (Fig. 1*C*), which are known to be located at the external dermis and the epidermis (3, 7, 8). In contrast, there were only a few DCs infiltrating the tumor cells after s.c. injection (Fig. 1*D*).

Tumor Cell Rejection after i.d. Injection. After s.c. injection, PRO tumor cells invariably yielded a tumor that continued to grow progressively. On the other hand, 11 of 18 rats (61%) that received PRO cells i.d. in the thoracic wall and all of the rats (15 of 15) that received PRO cells i.d. in the ear developed tumors that completely regressed in 2 or 3 weeks (Fig. 2*A*). Four weeks after i.d. or s.c. tumor cell injection in the thoracic wall, all of the rats were re-challenged against the same tumor cell line by a s.c. PRO tumor cell injection into the flank. All of the rats that rejected the tumor cells after i.d. injection were immunized, and no tumor was palpable at the site of the secondary challenge up to 6 weeks after the injection. In contrast, re-challenge produced PRO tumors in the flanks of all of the rats that developed PRO tumors after either s.c. (15 of 15) or i.d. (7 of 18) tumor cell injection at the thoracic site (data not shown). To demonstrate that this tumor rejection was mediated by the immune system, we injected PRO tumor cells i.d. in the thoracic wall (10 rats) or in the ear (5 rats) of nude rats. In all these nude rats tumor grew (Fig. 2*B*).

i.d. Injection of Tumor Cells Induces a Denser Infiltration of DCs at the Injection Site than s.c. Injection. To analyze the mechanisms of regression after i.d. injection, immunohistological analyses were performed on tumors on days 4 and 7 after i.d. or s.c. tumor cell injection into naïve rats (Fig. 3). At these times, tumor cells were easily identifiable according to their typical morphology and formation of poorly differentiated dense nodules. Tumors sites after s.c. injection (Fig. 3, s.c. column) were surrounded by a thin peritumoral halo containing a limited number of $ED1^+$ monocytes and immature DCs, sparse $ED2^+$ mature macrophages, nearly no $TCR^+/CD4^+$, and a few $TCR^+/CD8^+$ T cells, as well as some MHC class II^+ cells. In contrast, at day 4 after i.d. injection (Fig. 3, i.d. column), tumor nodules were densely infiltrated by $ED1^+$ and MHC-II^+ cells, which are a characteristic of rat DCs. At day 7, $ED1^+$ cells were replaced by a dense infiltration of $ED2^+$ mature macrophages within the tumor nodule. These macrophages were also $CD8^+$, a characteristic of activated rat macrophages (10). These $CD8^+$ cells were not T lymphocytes as most of the $CD8^+$ cells were not labeled with anti-TCR. Nevertheless, $CD8^+$ T cells were more abundant in the tumor site after i.d. injection.

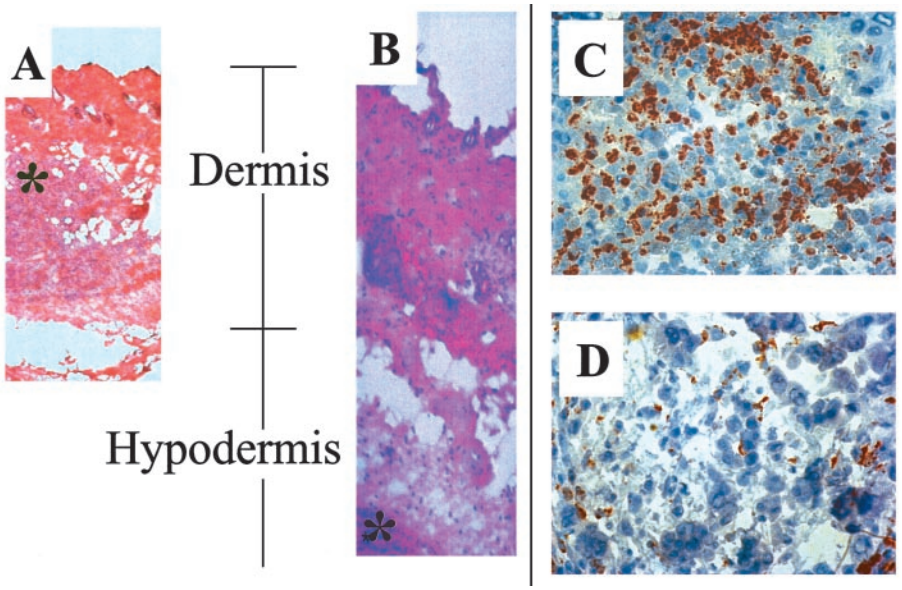

Fig. 1. Immunohistological analysis of cutaneous tissue. Histological and immunohistochemical analyses were performed on serial sections of rat skin just after i.d. (*A*, ×40) or s.c. (*B*, ×40) tumor cell injection using H&E labeling and 48 h after i.d. (*C*, ×250) or s.c. (*D*, ×250) tumor cell injection using ED-1 mAb that labeled immature DCs and monocytes.

i.d. Injection of FITC-labeled Tumor Cells Induces Migration of DCs, which Have Engulfed Fluorescent Tumor Cell Fragments, into the Draining Lymph Nodes. To follow the trafficking of tumor cell proteins during the initiation of the immune response, we labeled tumor cells with FITC. We have previously confirmed that FITC could induce a staining of tumor proteins and that FITC-labeled tumor proteins of an immunogenic tumor cell line could be engulfed by the immature tumor-infiltrating DCs, which then migrated to the draining lymph nodes (1). In this study, 4 days after i.d. injection of FITC-labeled PRO cells, low numbers of brightly fluorescent cells with dendritic morphology could be detected in the draining lymph nodes only after i.d. injection but not after s.c. injection using UV illumination (Fig. 4*A*) or fluorescence-activated cell sorting analysis (Fig. 4*B*). When analysis was restricted to gated larger cells, 3% of the larger cells were found to be fluorescent after i.d. injection, whereas no fluorescent cells were observed in the large cells recovered from the draining lymph nodes collected after s.c. injection of FITC-labeled PRO cells (Fig. 4*B*). To better characterize the migrated FITC-labeled cells, tumor-draining lymph nodes were dissociated and analyzed by flow cytometry. Nearly all FITC-labeled cells, recovered from lymph node draining sites of i.d. injected FITC-labeled PRO cells, had a dendritic morphology and expressed MHC class II molecules (Fig. 4*B*). These data strongly suggested that FITC-labeled cells found in the draining lymph nodes after i.d. injection of FITC-labeled PRO cells were DCs containing tumor cell proteins. T cells from draining lymph nodes were activated against PRO tumor cell Ags measured using proliferation (Fig. 4*C1*) and IFN-γ synthesis (Fig. 4*C2*) assays, only after i.d. tumor cell injection. Because of the moderate local swelling induced after an i.d. injection, we looked for synthesis of TNF-α. A significant amount of TNF-α mRNA was detected in tissues harvested 6 and 24 h after i.d. but not after s.c. injection (Fig. 4*D*).

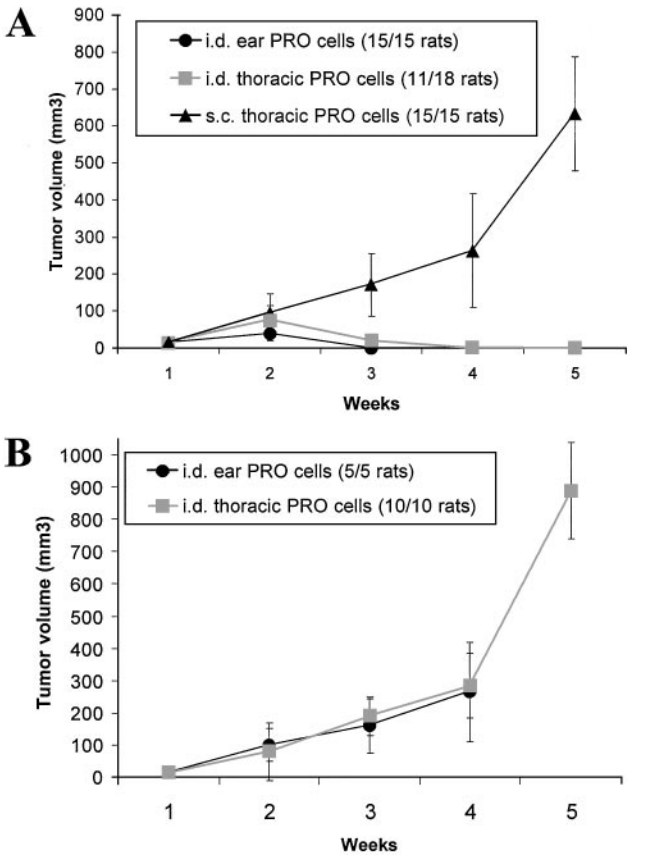

Fig. 2. Growth curves of the tumor after i.d. or s.c. tumor cell injection in naive rats. *A*, 10^6 PRO tumor cells were injected s.c. into the thoracic wall, i.d. in the thoracic wall, or i.d. in the ear of syngeneic BD-IX rats. All of the s.c. injections gave rise to progressive tumors, whereas all of the rats that had received i.d. PRO cells in the ear and 11 of 18 rats, which have received i.d. PRO cell into the thoracic wall, gave rise to tumors that completely regressed in 2–3 weeks. *B*, 10^6 PRO cells were injected i.d. in the thoracic wall or in the ear of nude rats. The curves represent the mean of two independent experiments. *Bars* indicate SD.

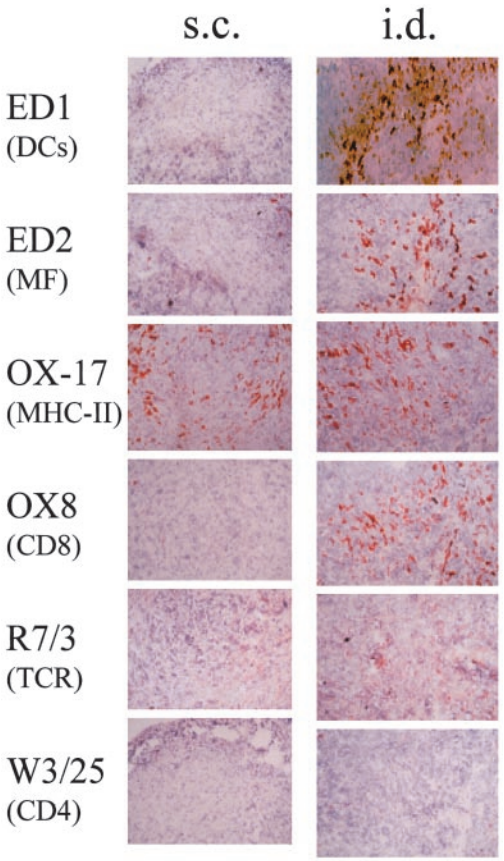

Fig. 3. Histological study of the tumor cell injection site. Animals were sacrificed 4 or 7 days after i.d. or s.c. tumor cell injection. Immunohistochemical analyses were performed on serial tumor sections, using mAbs that labeled monocytes and DCs (ED1), mature macrophages (ED2), MHC class II^+ cells (OX-17), $CD8^+$ T cells and macrophages (OX8), TCR^+ T cells (R7/3), and $CD4^+$ T cells (W3/25). Labeling of tumor slides after s.c. injection are in the column s.c., slides after i.d. injection are located in the column i.d. ($\times$150). Similar results were observed in three pairs of tumor injection sites after i.d or s.c. injection.

DISCUSSION

In this study, we demonstrate that the site of cell injection significantly influences the tumorigenicity and immunogenicity of an experimental tumor model. When injected i.d., normally nonimmunogenic tumor cells can induce an immune response and be rejected. It has been previously reported that i.d. injection of various tumor cell lines, including sarcoma and leukemia, led in a variable proportion to a transient tumor followed by tumor regression and sometimes immunization against additional challenge with the same cell line through alternate routes such as i.p. or s.c. (11–14). However, the mechanism of this antitumor immune response induced by i.d. tumor cell injection has not been precisely defined. This rejection is immune-mediated because tumor cells were not rejected after i.d. injection by the rats devoid of a functional immune system such as nude rats. Skin is composed of the epidermis and the dermis both rich in DCs and the hypodermis, which is continuous with the s.c. tissue poor in antigen-presenting cells. The high concentration of DCs in the

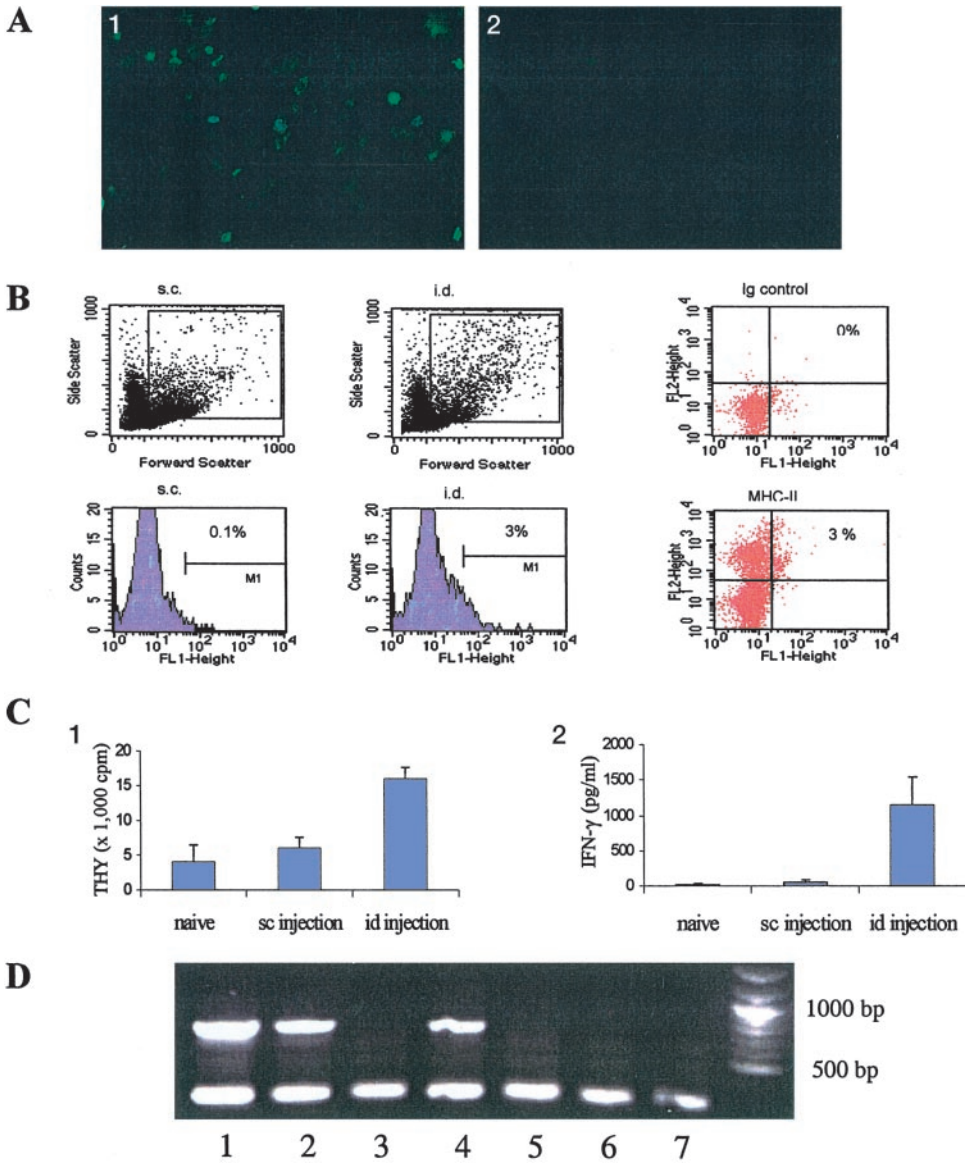

Fig. 4. Analyses of the DCs and the T cells recovered in the draining lymph nodes after i.d. or s.c. tumor cell injection. Four days after s.c. or i.d. injection of FITC-labeled PRO cells into the thoracic wall, the draining lymph nodes of 5 rats were pooled and disaggregated in a suspension. In very concentrated cell suspensions, a few cells were FITC positive in the suspension obtained from the draining lymph nodes only after i.d. injection (Fig. 4*A1*) and not in the lymph nodes cell suspension obtained after s.c. injection (Fig. 4*A2*). A cytometer analysis confirmed these results (Fig. 4*B*). An analysis of only the larger cells (gated) demonstrated that ~3% of these large cells were FITC positive (FL1-Height). Double labeling showed that nearly all of the fluorescent cells expressed MHC class II molecules (FL2-Height). T cells recovered from the draining lymph nodes 4 days after i.d. or s.c. PRO tumor cell injection were cocultured in triplicate with mitomycin-C-treated PRO tumor cells (1×10^5 cells/ml). We compared thymidine incorporation into T cells from draining lymph nodes after i.d. or s.c. tumor cell injection or without injection 3 days after the beginning of the mixed culture (Fig. 4*C1*). After 3 days of coculture, supernatants were collected and IFN-α concentrations was measured using ELISA kit (Fig. 4*C2*). Reverse transcription-PCR was performed with RNA isolated from tumor cell injection site, 6, 24, and 48 h after i.d. or s.c. injection. A semiquantitative assay was performed by coamplifying a fragment of GAPDH and TNF-α (Fig. 4D). *Lane 1:* RNA from activated macrophages as positive control; *Lanes 2, 4,* and *6:* RNA isolated, respectively, 6, 24, and 48 h after i.d injection; *Lanes 3, 5,* and *7:* RNA isolated 6, 24, and 48 h after s.c. injection.

dermis may facilitate capture of tumor antigens when nonimmunogenic tumor cells are i.d. injected (7–8). In contrast, an immunogenic tumor has been reported to grow when grafted to an extra lymphatic site. The loss of immunogenicity is believed to be attributable to the inability of tumor-antigen loaded DCs to reach the lymph nodes (15). Taken together, these data support the hypothesis that the immunogenicity of injected tumor cells depends on the numbers of DCs at the injection site. A slight modification of the depth of injection could influence the tumorigenicity and immunogenicity, and this fact, associated with the difficulty of i.d. injection in mice because of the thinness of the dermis, may explain the repeatedly observed phenomenon that only a certain proportion of mice of the same experimental group develop protection after tumor vaccination (11–14). This problem could be resolved by choosing an injection site where there is no s.c. tissue like the ear.

Increased tumor cell death induced by i.d. injection may explain the immunogenicity of this injection route with facilitating the capture of tumor Ags by DCs. Using a terminal deoxynucleotidyl transferase-mediated nick end labeling method, we did not find more apoptosis after i.d. than s.c. injection in the tumor site injection (data not shown).

To present antigens to naïve T lymphocytes, antigen-loaded DCs have to migrate into the draining lymph nodes. In this work, we used PRO cells, which had been previously labeled *ex vivo* with FITC (1), to visualize the migration of tumor cell proteins after i.d. injection of covalently FITC-labeled PRO cells. Four days after this injection, fluorescent DCs, identifiable by their morphology and MHC class II^+ phenotype, were found in the draining lymph nodes. By contrast, no fluorescent DCs were observed in the draining lymph nodes after s.c. injection of FITC-labeled PRO cells.

To present tumor antigens efficiently to T cells in the lymph nodes, immature DCs that infiltrate the tumor have to differentiate into mature DCs, which have reduced endocytosis capacity but overexpress MHC-peptide immunogenic complexes and costimulatory signals such as B7-1 and B7-2, on their plasma membrane. DC maturation can be induced by various inflammatory cytokines such as interleukin 1β and TNF-α (2). In addition, the proinflammatory cytokine TNF-α has been shown to be critical for inducing DC migration from the dermis to the lymph nodes (16, 17). Interestingly, only after i.d. but not after s.c. injection of PRO cells, local transcription of TNF-α mRNA was observed. Besides its role in the maturation of DCs and their migration into draining lymph nodes, TNF-α can

also attract immature DCs by inducing keratinocytes to secrete CCL20, the most potent known chemokine for attracting immature DCs (18). In the draining lymph nodes after i.d. tumor cell injection, DCs were potent tumor antigen-presenting cells as they induced specific T-cell proliferation. Activated T cells secrete IFN-γ, which could activate macrophages, the effector cell directly responsible for PRO tumor cell killing (19).

In conclusion, in contrast to s.c. PRO tumor cell injection, which gives rise to progressive and lethal tumors, i.d. injection of the same cell line induces an efficient antitumor immune response, leading to complete regression. Tumor regression correlated with a high density of DCs in the dermal tissue, which may facilitate the engulfment of tumor Ags. Furthermore, tumor cell i.d. injection induces synthesis of proinflammatory TNF-α, which may enhance DC maturation into fully efficient antigen-presenting cell and migration into the draining lymph nodes to activate tumor antigen-specific T cells. These data have important implications both for the interpretation of experimental results in which tumor cells are used and for the design of clinical trials using tumor vaccines. The richness of DCs in skin should make i.d. site a preferable site for injection of modified tumor cells in experimental and clinical trials using tumor vaccines.

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