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Advances in Brief

Interleukin 10 Transfected into Chinese Hamster Ovary Cells Prevents Tumor Growth and Macrophage Infiltration[1]

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Abstract

Expression of cytokines in tumor cells provides a sensitive modality to analyze the consequences of local cytokines *in vivo* on tumor infiltrating cells and tumorigenicity. We have transfected Chinese hamster ovary (CHO) cells with an interleukin 10 (IL-10) expression vector. CHO-IL10 cells although unaltered with respect to their *in vitro* growth lost tumorigenicity, both in nude and in SCID mice and in an IL-10 dose dependent manner. In addition, CHO-IL10 cells suppressed the growth of equal numbers of coinjected but not of contralaterally injected CHO cells. Immunohistology with anti-CR3/Mac-1 and anti-Mac-3 monoclonal antibodies revealed that CHO tumors were substantially infiltrated by macrophages. However, in CHO-IL10 tumors macrophages were virtually absent within the tumor tissue. Our results suggest that IL-10 indirectly suppresses tumor growth of certain tumors by inhibiting infiltration of macrophages which may provide tumor growth promoting activity.

Introduction

Tumors are often infiltrated by macrophages. In some well-characterized systems tumor cells secrete macrophage chemotactic factors, yet the role of macrophages in tumorigenesis is still an enigma. Due to the many functions and the varying activation states of macrophages, it has been proposed that they may exert growth promoting and suppressive properties (for review see Ref. 1). Direct tumoricidal activity of appropriately activated macrophages has been demonstrated *in vitro* (2–5) and recent results suggest their contribution during rejection of cytokine gene transfected tumors (6–11). Less clear and mostly indirect is the evidence for tumor promoting effects of macrophages; tumor infiltrating macrophages showed few tumoricidal activity and even promoted tumor cell growth *in vitro* (12, 13). Macrophage functions such as induction of angiogenesis and fibrin deposition have also been held responsible for support of tumor growth *in vivo* (14, 15). IL-10[3] is produced by and acts on a number of cell types (*e.g.*, T-cells, B-cells, and monocytes/macrophages; for review see Ref. 16). A key role of IL-10 in the regulation of immune responses may be its deactivating effects on macrophages. Thus, IL-10 down-regulates major histocompatibility complex class II expression and suppresses production of reactive nitrogen oxides and several proinflammatory cytokines (IL-1α, IL-6, IL-8, tumor necrosis factor, granulocyte-macrophage and granulocyte colony stimulating factors) by monocytes/macrophages (16–21). To analyze the effect of IL-10 on tumor infiltrating macrophages and tumorigenicity we expressed the IL-10 gene in CHO cells which form tumors with a strong macrophage infiltrate.

Materials and Methods

Tumor Cells. CHO cells obtained from the ATCC (No. CCL 61) were passaged in a BALB/c-*nu/nu* mouse before gene transfer. Reisolated CHO cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics.

Transfection of Tumor Cells. The mouse IL-10 complementary DNA was isolated by reverse PCR with primers which allowed amplification of the entire coding region (22) and were supplemented with *SalI* palindromic sequences (5′-AGAGTCGACCATCATGCCTGGCTCAGCA-3′, 5′-GCAGTCGACTT-AGCTTTTCATTTTGATCATCA-3′). Substrate for PCR was RNA of concanavalin A activated spleen cells. Spleen cell activation, RNA extraction, complementary DNA synthesis, and PCR were done as described (9). The PCR product was cloned into the *SalI* restriction site of pBMGneo (23) resulting in pBMGneo.IL-10. Cells were transfected with plasmids pBMGneo.IL10 or pBMGneo by electroporation using a Gene-Pulser (Bio-Rad Laboratories, Richmond, CA) under the following conditions: 5×10^6 cells in 0.5 ml Dulbecco's phosphate buffered saline (without Ca^{2+} and Mg^{2+}) were pulsed with 875 V/cm, $\tau = 5$ ms. Transfected cells were selected for G418 (1 mg/ml) resistance and clones were established.

Assays for IL-10 Activity. IL-10 activity was detected by its ability to enhance the IL-4 induced proliferation of mast cell line D36 (24). D36 cells were cultured at a density of 1×10^3 cells/well in 100 μl medium in the presence of mouse IL-4 (8 units/ml) and serial dilutions of conditioned medium of transfectants. After 24 h cells were pulse labeled with 0.1 μCi [^{3}H]-thymidine (specific activity, 5 Ci/mmol) for 18 h. Cells were harvested onto glass fiber plates, and [^{3}H]thymidine incorporation was quantified by scintillation counting. Specificity for IL-10 was confirmed by addition of neutralizing anti-mIL10 mAb SXC1 (25). One unit of IL-10 was defined as the amount required to obtain half-maximal proliferation. Additionally, IL-10 production was determined by an enzyme linked immunosorbent assay with mAbs JES5-2A5 and SXC1 (both from Pharmingen, San Diego, CA). Transfectant medium was conditioned for 48 h at a cell concentration of 2.5×10^5/ml.

Analysis of Tumor Growth. Cells were harvested by trypsinization, washed with Dulbecco's phosphate buffered saline, and injected s.c. in a volume of 0.2 ml into 6–8-week-old female mice (BALB/c-*nu/nu*, CB17 SCID; obtained from Bomholtgard, Ry, Denmark). Tumor size was determined as described (26). Mice bearing a tumor >1 cm in diameter were considered as positive. In some experiments, tumor tissue was excised at different time points after tumor cell injection (days 2, 4, and 5) for immunohistology and analysis of IL-10 activity after *in vivo* growth similarly to described procedures (26).

Immunohistology. Immunohistology of tumor tissue was done as described (26), using rat anti-mouse CR3 (Mac-1) mAb M1/70 (ATCC TIB 128) and anti-Mac-3 mAb M3-84 (ATCC TIB 168) or isotype matched control mAb.

Results

The mouse IL-10 expression vector pBMGneo.IL10 was constructed and used to transfect CHO cells. As control CHO cells were transfected with plasmid pBMGneo. G418^R cells were selected and cloned, and IL-10 activity was analyzed in culture supernatants (Table 1). Parental CHO and mock-transfected CHO-neo-clones 1, 2, 3, and

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[3] The abbreviations used are: IL, interleukin; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; mAb, monoclonal antibody; ATCC, American Type Culture Collection.

Table 1 *IL-10 expression in transfected CHO cells*

Cell line	Expression vector	IL-10 activity (units/ml)[a]
CHO		
CHO-neo (clone 1, 2, 3, 4)	pBMGneo	
CHO-IL10.2	pBMGneo.IL10	20
CHO-IL10.8	pBMGneo.IL10	250
CHO-IL10.9	pBMGneo.IL10	150

[a] IL-10 activity secreted by 2.5×10^5 cells/ml during 48 h, as determined by mast cell proliferation assay or enzyme linked immunosorbent assay. Results represent the average of eight independent experiments, each of which was performed in triplicate.

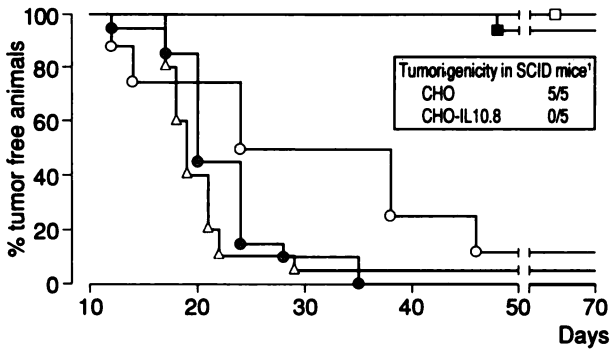

Fig. 1. Tumorigenicity of CHO (△), CHO-neo (⊙), CHO-IL10.2 (○), CHO-IL10.8 (■), and CHO-IL10.9 (□) cells injected at a dose of 5×10^6 cells s.c. into BALB/c-*nu/nu* mice. Twenty CHO, 20 CHO-neo (combined results of each 5 mice given injections of CHO-neo clones 1, 2, 3, and 4), 8 CHO-IL10.2, 19 CHO-IL10.8, and 8 CHO-IL10.9 cell injected mice were analyzed. The combined results of several experiments are shown. [1]Inset, number of SCID mice with tumor/number of mice given s.c. injections of 5×10^6 of the indicated cells. Mice were observed for 50 days.

4 did not produce any IL-10. CHO-IL10.2; CHO-IL10.8, and CHO-IL10.9 cells produced between 20 and 250 units/ml. The *in vitro* growth kinetics had not changed in any of the transfectants and exogenous IL-10 did not affect the growth of CHO cells *in vitro* (data not shown). Tumorigenicity of CHO-IL10.2, CHO-IL10.8, and CHO-IL10.9 cells was compared to that of parental CHO or mock transfected cells by s.c. injection into nude mice (Fig. 1). Ninety-seven % of the mice (39 of 40) given injections of non-IL-10 producing cells developed a tumor. In contrast, in 96% of the mice CHO-IL10.8 and CHO-IL10.9 cells failed to grow as tumor (26 of 27). Likewise, CHO but not CHO-IL10.8 cells gave rise to tumors in SCID mice (see Fig. 1, *inset*). Mice given injections of low IL-10 producing CHO-IL10.2 cells developed a tumor with a considerable delay showing that the strength of tumor suppression correlated with the amount of secreted IL-10. Recultivated tumors from these mice demonstrated that they had stopped IL-10 expression during *in vivo* growth in all cases (7 of 7). To address the question of whether the tumor suppressive effect of IL-10 acted locally and indirectly, the same numbers of IL-10 producing and parental or mock transfected CHO cells were either mixed before injection or injected contralaterally and tumor growth was observed. Table 2 (Experiment 1) shows that CHO-IL10.8 cells were unable to suppress tumor growth of contralaterally injected CHO cells. However, coinjection of both cell types at the same site resulted in suppression of tumor growth of parental CHO and CHO-neo.3 cells (Table 2, Experiment 2). Since CHO-IL10 cells did not grow in nude, SCID, and also in asialo-G_{M1} antiserum treated nude mice (not shown), neither T-cells, nor B-cells, nor natural killer cells seemed to be responsible for this effect. To further investigate the involved mechanism, an immunohistological evaluation of tumor tissue was performed. Already visible on day 2 and 5, CHO tumors were heavily infiltrated by Mac-1^+ cells (Fig. 2*a*) which were evenly distributed within the tumor tissue. Due to their morphology and because of a similar staining pattern with an anti-Mac-3 mAb in serial tissue sections, these cells were identified as macrophages. In CHO-IL10 tumors, however, Mac-1^+ cells were virtually absent within the tumor tissue and were found only in the surrounding area of the tumor which already contained necrotic areas (Fig. 2*b*). Furthermore, the macrophages in CHO-IL10 tumors showed decreased MHC class II expression in comparison to macrophages in CHO tumors (not shown). Thus, macrophages in IL-10 producing CHO tumors are less abundant than in control tumors and/or carry a marker for deactivation.

Discussion

We have shown that IL-10 expression by CHO cells abrogated their tumorigenicity. Several lines of evidence attribute this effect to IL-10: (*a*) the suppressive effect of IL-10 on CHO tumor growth was observed in several clones from different transfections; (*b*) it correlated with the amount of IL-10 secretion; (*c*) CHO-IL10 cells suppressed also the growth of coinjected parental cells; and (*d*) a selective pressure against IL-10 production consisted on CHO-IL10 cells *in vivo* because the late outgrowth of low IL-10 producing CHO cells was always associated with loss of IL-10 production although the cells stably expressed IL-10 *in vitro* over that period. No T-, B-, or natural killer cells were needed for tumor suppression and no other cells infiltrating IL-10 producing tumors (eosinophils, granulocytes; not shown) could be detected immunohistologically. Therefore, it seems that IL-10 did not induce an antitumor response as observed with a number of cytokines transfected into tumor cells (27). The only immunological effect we could detect in CHO-IL10 tumors was the lack of a macrophage infiltrate in the tumor tissue. Macrophages were observed only in the surrounding area of the tumor which was already necrotic 3 days after injection of the cells. Additionally, macrophages surrounding CHO-IL10 tumors expressed less major histocompatibility complex class II antigens than those in CHO tumors (demonstrated by immunohistology; data not shown) arguing for a deactivating effect of IL-10 on macrophages *in vivo*, similar as observed *in vitro* (17). Furthermore, coinjection of J558L plasmacytoma cells which form tumors with a very low or absent macrophage infiltrate (10) with CHO-IL10 cells did not result in a growth retardation of J558 tumors (not shown). The failure of CHO-IL10 cells to grow as tumor may be explained by their need of macrophages or their products to grow *in vivo*. However, it is not currently known which macrophage activity CHO cells need for *in vivo* growth or whether the inability of macrophages to infiltrate the tumor is a direct or indirect effect of IL-10. The observation that no initial tumor formation was visible and tumor necrosis was observed very rapidly indicates that CHO-IL10 cells were impaired to establish early tumor growth. In this regard, it is important to note that tumor infiltrating macrophages may provide growth promoting activity for some tumors *in vitro* (1), that tumor

Table 2 *CHO-IL10 cells suppress tumor growth of coinjected but not contralaterally injected CHO cells*

Five $\times 10^6$ of the indicated cells were injected s.c. into BALB/c-*nu/nu* mice. Tumor free animals were observed for 50 days.

Cell line	No. of mice with tumor/ no. of mice in experiment
Experiment 1	
CHO	5/5
CHO-IL10.8	1/5
CHO/CHO-IL10.8 (contralateral)	5/5[a]
Experiment 2	
CHO	5/5
CHO-neo.3	5/5
CHO-IL10.8	0/4
CHO-IL10.9	0/5
CHO-IL10.8/CHO-neo.3	1/5
CHO-IL10.8/CHO	0/6
CHO-IL10.9/CHO-neo.3	0/6

[a] Tumors at the site of CHO cell injection.

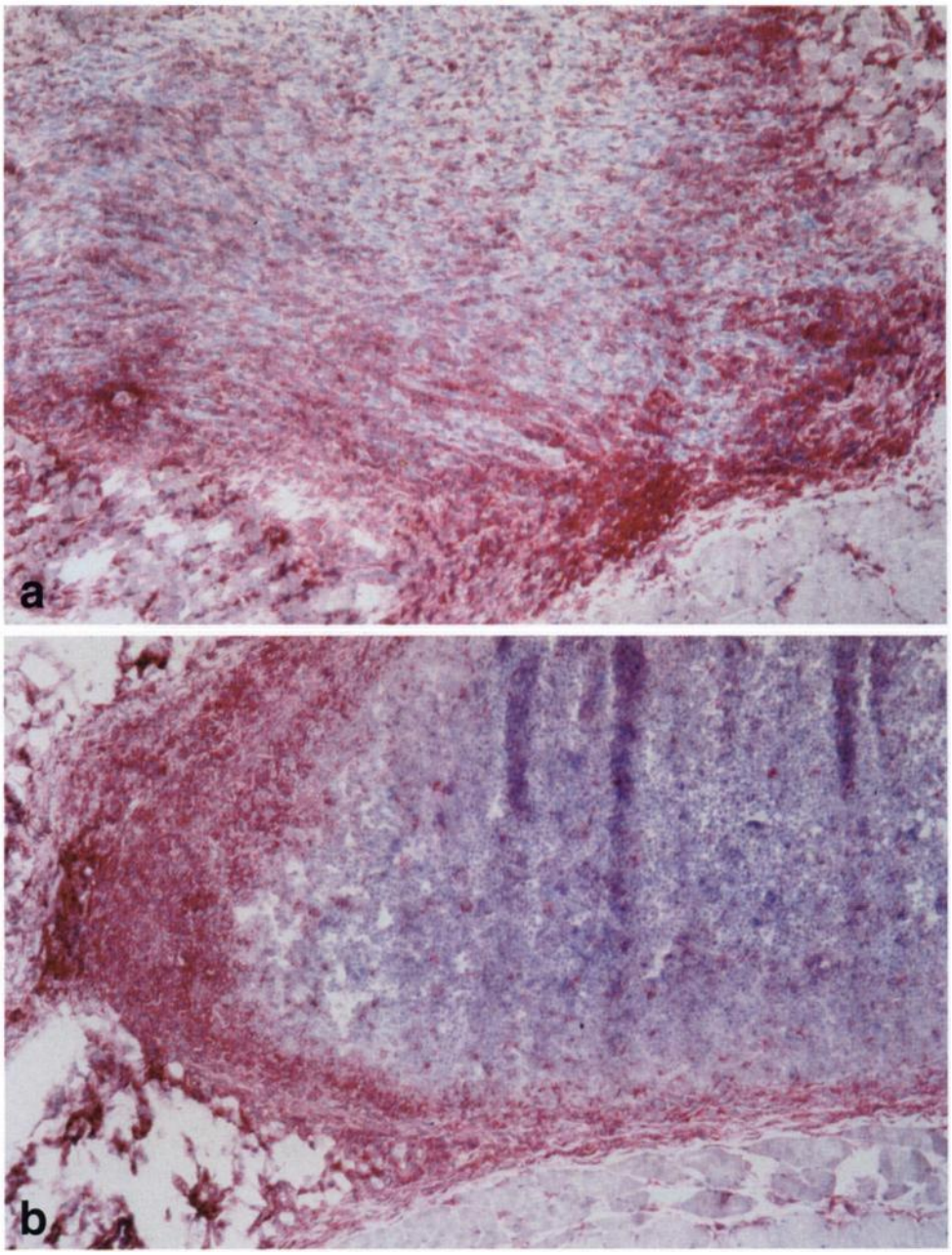

Fig. 2. Immunohistochemical staining with anti-CR3 (Mac1) mAb of tumor tissue from nude mice 4 days after s.c. injection of 5×10^6 CHO-neo (*a*) and CHO-IL10 (*b*) cells. A similar staining pattern was obtained in serial tissue sections with an anti-Mac 3 mAb. Note that in CHO-neo tumor (*a*) macrophages have infiltrated the tumor tissue whereas in CHO-IL10 tumor (*b*) they are absent within but abundantly surround the tumor tissue. The results were confirmed with tumors from several mice and at different time points (see "Materials and Methods"). ×400.

associated macrophages can induce neovascularization *in vivo* (14) and, as recently shown, that expression of the vascular endothelial growth factor in CHO cells confers a growth advantage of these cells in nude mice (28). The latter effect has been explained by a direct angiogenic property of vascular endothelial growth factor which supports growth of CHO cells *in vivo*. These results together with our findings suggest that vascularization and concomitant tumor growth of CHO cells in nude mice is paracrinely stimulated by the host. In what respect IL-10 acts antiangiogenic indirectly mediated by inhibition of tumor associated macrophages has to be further analyzed.

Like CHO-IL10 cells, IL-4 (9) and MCAF (7) transfected CHO cells were strongly suppressed with respect to their *in vivo* growth in nude mice. However, in these cases the tumoricidal activity of the macrophages was at least in part responsible for this effect. Therefore, the local cytokine environment very much determines the activation stage of macrophages *in vivo*.

Due to the immunosuppressive and antiinflammatory effects of IL-10 one might think that some tumors use this strategy to escape immuno-surveillance. Therefore, any judgment about immunotherapeutic potential of IL-10 will depend on clear demonstrations of whether and which tumor cell type requires growth promoting activity by the host that can be suppressed by IL-10.

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