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Suppression of Tumor Formation In Vivo by Expression of the *JE* Gene in Malignant Cells

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The early growth response gene *JE* encodes a monocyte chemoattractant, MCP-1. The JE/MCP-1 protein attracts and stimulates human monocytes and induces monocyte-mediated inhibition of tumor cell growth in vitro. Expression of human or murine JE/MCP-1 in Chinese hamster ovary (CHO) cells completely suppressed their ability to form tumors in nude mice. Coinjection of JE/MCP-1-expressing cells with nonexpressing CHO cells or with HeLa cells also prevented tumor formation. Since JE/MCP-1 expression had no discernible effect on the transformed phenotype of these cells in vitro, the suppressive effect depends on host animal factors. These factors are likely to be components of the inflammatory response, because JE/MCP-1-expressing cells elicited a predominantly monocytic infiltrate at the site of injection. Our results suggest that JE/MCP-1 protein may be useful in cancer therapy.

The *JE* gene is a platelet-derived growth factor-inducible competence or early-response gene first identified in mouse 3T3 cells (3). Sequence and expression analysis showed that unlike other early response genes, such as c-*myc*, c-*fos*, and *c-jun*, the murine *JE* gene encodes a secreted glycoprotein with cytokinelike properties (11, 20). The human homolog of murine *JE* has been cloned (22), and the predicted amino acid sequence of its protein is identical to that of a monocyte chemoattractant, MCP-1 (32, 33) (also called MCAF [6, 16] and SMC-CF [7, 27]). The JE/MCP-1 protein is structurally related to the members of a large, recently identified family of low-molecular-weight secreted proteins that appear to be involved in the inflammatory response (14, 22, 29). The genes for many of these proteins, including human JE/MCP-1, are clustered on chromosome 17q11.2-12 (4, 9, 21) or mouse chromosome 11 (23a, 28). These genes are also related to the genes that encode another family of cytokines, whose members include the neutrophil activator NAP-1/IL-8 (18, 23, 31), many of which cluster at 4q12-21 (8, 15, 19).

JE/MCP-1 exerts several effects specifically on monocytes. Both natural and recombinant JE/MCP-1 proteins are potent chemoattractants for human monocytes in vitro (16, 32), and purified recombinant JE/MCP-1 can stimulate an increase in cytosolic free calcium and the respiratory burst in monocytes (19a, 34). Purified natural JE/MCP-1 has also been reported to activate monocyte-mediated inhibition of tumor cell growth, but not tumor cell killing, in vitro (16). On the basis of its in vitro properties, JE/MCP-1 might be expected to attract monocytes in vivo and, perhaps, to activate their anti-tumor cell properties. Here, we tested whether JE/MCP-1 expression can suppress tumor formation in vivo. We found that malignant cells engineered to express JE/MCP-1 no longer formed tumors in nude mice and that coinjection of these cells with malignant cells not expressing JE/MCP-1 also suppressed tumor formation. Our results suggest that JE/MCP-1 induces monocyte-mediated tumoricidal activity in vivo and that this protein may find an important therapeutic use as an antitumor agent.

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MATERIALS AND METHODS

Cell culture. DUKXB-11 cells (26) (dihydrofolate reductase mutant Chinese hamster ovary [CHO] cells) were grown in the alpha modification of minimal essential medium (MEM-α) without ribonucleosides and deoxyribonucleosides, supplemented with 10% bovine calf serum (BCS) and 10 μg each of adenosine, deoxyadenosine, and thymidine per ml (MEM-α–BCS–AAT) (10). HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum and antibiotics.

Transfections and MTX selection. DUKXB-11 cells were grown in 10-cm-diameter culture dishes as described above. At 60% confluence, medium was removed and replaced with fresh MEM-α–BCS–AAT supplemented with 3 μg of Polybrene per ml and incubated overnight (1). On the next day, each dish was given 3 ml of fresh MEM-α–BCS–AAT with 83 ng of plasmid DNA per ml and 10 μg of Polybrene per ml. The cells were incubated at 37°C for 6 h with rocking every 90 min. The medium was then removed and replaced with 5 ml of fresh MEM-α–BCS–AAT containing 30% dimethyl sulfoxide for exactly 4 min. The medium was removed, and the cells were washed once with MEM-α and given 10 ml of MEM-α–BCS–AAT. The cells were incubated at 37°C for 48 h, trypsinized, replated into four culture dishes (10-cm diameter) in nucleoside- and deoxynucleoside-free MEM-α supplemented with 10% dialyzed BCS, and refed with this medium every 3 days. Two independent transfections were performed with pXM (30), pXM-JE10 (murine *JE* cDNA [20] in the sense orientation), pXM-JE1 (murine *JE* cDNA in the antisense orientation), and pXM-hJE34 (human *JE* cDNA [22]). Colonies from each independent transfection that grew in ribonucleoside- and deoxyribonucleoside-free medium were trypsinized and combined. Stepwise selection was carried out with 0.02, 0.1, 0.5, 2.0, 10.0, and 100.0 μM methotrexate (MTX). At each concentration, surviving colonies were trypsinized and pooled.

Protein analysis. Confluent cell cultures were incubated in methionine-free MEM-α with 2% dialyzed BCS for 45 min and then changed to 0.5 ml of the same medium with 500 μCi of [^{35}S]methionine (DuPont NEN, Boston Mass.). Cells were incubated at 37°C for 4 h, after which the medium was collected, made 1 mM in phenylmethylsulfonyl fluoride,

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centrifuged to remove cells and debris, and stored at −70°C. Immune precipitations using anti-JE/MCP-1 serum were performed (22), and the results were analyzed by electrophoresis through a sodium dodecyl sulfate-containing 17% polyacrylamide gel.

Soft agar colony formation assay. Five thousand cells were suspended in MEM-α containing 10% dialyzed BCS, 0.3% agar, and the appropriate concentration of MTX. While still molten, this suspension was distributed on a gelled 4-ml underlayer of MEM-α containing 10% dialyzed BCS, 0.6% agar, and the appropriate concentration of MTX in a 60-mm-diameter culture dish. Cells were fed with three drops of fresh medium every 5 days. After 14 days, colonies consisting of greater than 50 cells were counted.

MCA. Confluent monolayers of CHO cells were incubated in serum-free MEM-α for 24 h, after which the medium was centrifuged to remove cells and debris and the remaining adherent cells were trypsinized and counted. Fresh human peripheral blood mononuclear cells were purified from the blood of volunteer donors by centrifugation on a cushion of Ficoll-Hypaque (Pharmacia, Piscataway, N.J.). Cells at the interface were washed twice in Gey's balanced salt solution with 2% bovine serum albumin and then suspended at 4×10^6/ml in Gey's balanced salt solution with 0.2% bovine serum albumin. Medium from CHO cells was serially diluted in Gey's balanced salt solution with 0.2% bovine serum albumin, and monocyte chemoattractant activity (MCA) was measured in a 48-well microchamber apparatus (5). The concentration of MCA in CHO cell medium was defined as the reciprocal of the dilution showing half-maximal activity (32).

Nude mouse injections. Cells were suspended in 0.2 ml of phosphate-buffered saline and injected subcutaneously into 4-week-old male Swiss *nu/nu* mice. The mice were monitored daily for tumor growth. Tumor volume was derived by multiplying the values of three perpendicular diameters.

RESULTS

Engineered expression of JE/MCP-1 in CHO cells. To create malignant cells expressing JE/MCP-1, we transfected dihydrofolate reductase deletion mutant CHO cell line DUKXB-11 (26) with expression vector pXM (30), containing a variety of *JE* cDNA species. We then achieved high levels of JE/MCP-1 protein expression in stably transfected lines by MTX-induced DNA amplification (10). Figure 1 shows JE/MCP-1 protein expression in independently derived cell lines selected for resistance to 2 or 10 μM MTX. No detectable JE/MCP-1 protein was secreted from cell lines transfected with pXM alone (cell lines 0A-2 and 0B-2; cell line 0A-10 was derived from 0A-2) or with pXM containing murine *JE* cDNA in the antisense orientation (1A-2 and 1B-2). Considerable JE/MCP-1 protein was secreted by cell lines transfected with murine *JE* cDNA in the sense orientation (10A-2 and 10B-2; 10A-10 was derived from 10A-2) and human *JE* cDNA (hJEC-10). Cell line hJEC-100, derived from hJEC-10 by selection for resistance to 100 μM MTX, also secreted human JE/MCP-1 protein (data not shown).

Murine *JE* cDNA directs CHO cells to secrete a microheterogeneous protein with an M_r of 27,000 to 39,000, similar to the natural protein and the protein expressed in a COS cell expression system (20). We have previously demonstrated that nearly half of the apparent M_r is due to O-linked glycosylation (20). Human JE/MCP-1 proteins expressed in CHO cells are also similar to native and COS cell-expressed JE/MCP-1α (M_r, 15,000) and JE/MCP-1β (M_r, 11,000) (22).

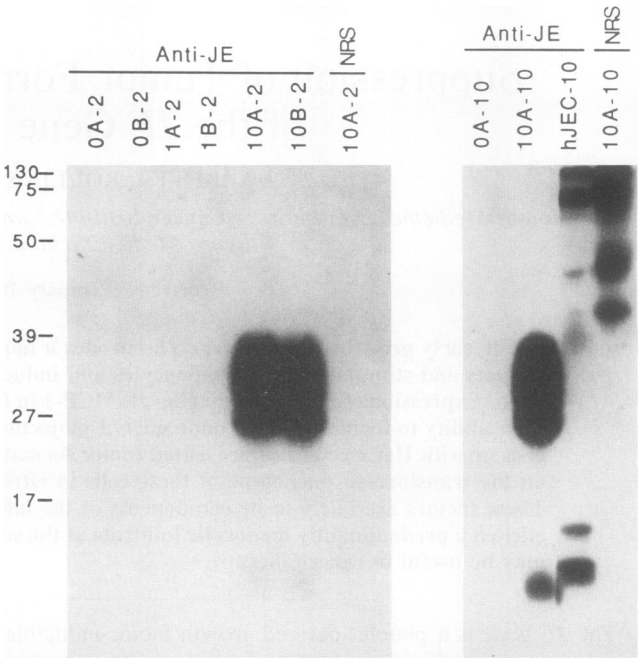

FIG. 1. Expression of JE/MCP-1 in transfected CHO cell lines. DUKX-B11 cells were transfected with pXM alone (0A-2, 0B-2, and 0A-10), pXM-JE1 (1A-2 and 1B-2), pXM-JE10 (10A-2, 10B-2, and 10A-10), or pXM-hJE34 (hJEC-10 and hJEC-100 [see text]). Pooled colonies from each independent transfection were selected in stepwise fashion for resistance to 2 μM MTX (0A-2, 0B-2, 1A-2, 1B-2, 10A-2, and 10B-2) or 10 μM MTX (0A-10, derived from 0A-2; 10A-10, derived from 10A-2; and hJEC-10). Cells were radiolabeled with [^{35}S]methionine, and secreted proteins were analyzed by immune precipitation using anti-JE serum as described in Materials and Methods (22). Murine JE/MCP-1 appears as a microheterogeneous band with an M_r of 27,000 to 39,000, and human MCP-1/JE consists of two bands with M_rs of 11,000 (JE/MCP-1β) and 15,000 (JE/MCP-1α). Molecular size markers are indicated in kilodaltons. NRS, normal rabbit serum.

The MCAs secreted by these cell lines were determined as described in Materials and Methods. They were 1,415 U/24 h/10^6 cells for 10A-2, 1,079 U/24 h/10^6 cells for 10B-2, 3,008 U/24 h/10^6 cells for 10A-10, 54 U/24 h/10^6 cells for hJEC-10, and 692 U/24 h/10^6 cells for hJEC-100. JE/MCP-1-nonexpressing cells secreted 10 to 30 U/24 h/10^6 cells. The increased MCA secreted by murine JE/MCP-1-expressing lines is due to increased JE/MCP-1 protein in the medium, not to higher specific activity. Murine JE/MCP-1 appears to be more stable than human JE/MCP-1 in culture, perhaps because of its more extensive glycosylation (20).

Effect of JE/MCP-1 expression on the malignant phenotype of CHO cells. By several criteria, JE/MCP-1 expression did not alter the transformed phenotype of CHO cells in vitro. (i) While the doubling times of the independently derived cell lines varied considerably (Table 1), the average doubling time of all of the JE/MCP-1-expressing lines (25.1 ± 5.7 h [standard deviation]) was nearly identical to the average doubling time of the JE/MCP-1 nonexpressors (26.4 ± 5.7 h). (ii) JE/MCP-1 expression did not alter the transformed morphology of the CHO cells. (iii) All of the cell lines formed colonies in soft agar (Table 1).

In vivo, however, JE/MCP-1 expression led to a striking difference in behavior (Table 1). All but one (0B-2) of the JE/MCP-1-nonexpressing cell lines formed large subcutane-

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TABLE 1. Properties of transfected CHO cell lines[a]

Cell line	JE cDNA	Doubling time (h)	Mean no. (±SD) of soft agar colonies/25 mm²[b]	No. of cells injected	Tumors[c]
JE/MCP-1 nonexpressors					
0A-2	None	24.2	91 ± 14.0	2×10^7	1/1
0A-2	None	24.2	91 ± 14.0	1×10^7	2/2
0A-2	None	24.2	91 ± 14.0	2×10^6	3/4
0A-10	None	20.4	32 ± 1.9	1×10^7	2/2
0B-2	None	19.0	73 ± 2.6	1×10^7	0/4
1A-2	Antisense	30.7	75 ± 14.5	8×10^6	2/2
1B-2	Antisense	31.2	74 ± 1.7	1×10^7	1/2
JE/MCP-1 expressors					
10A-2	Murine	28.8	72 ± 7.3	2×10^7	0/1
10A-2	Murine	28.8	72 ± 7.3	1×10^7	0/2
10A-2	Murine	28.8	72 ± 7.3	2×10^6	0/4
10B-2	Murine	17.8	86 ± 5.1	1×10^7	0/2
10A-10	Murine	30.0	129 ± 6.8	1×10^7	0/2
hJEC-10	Human	28.8	32 ± 6.8	1×10^7	0/2

[a] CHO cells transfected with pXM, pXM-JE10, pXM-JE1, or pXM-hJE34 were selected for resistance to either 2 or 10 μM MTX. The indicated numbers of cells were tested for the ability to form tumors after subcutaneous injection into nude mice. Each cell line was tested in triplicate for its ability to form colonies in soft agar as described in Materials and Methods.
[b] Number of colonies consisting of >50 cells.
[c] Number of animals with tumors/number of animals injected.

ous tumors that appeared within 3 weeks of injection into nude mice. In contrast, all of the JE/MCP-1-expressing lines, including the human JE/MCP-1-expressing lines, formed no tumors for as long as 10 months after injection. At autopsy, there was no microscopic evidence of residual tumors in animals that received JE/MCP-1-expressing cells.

Coinjection of MCP-1/JE expressors and nonexpressors. These observations suggested the possibility that JE/MCP-1-secreting cells attracted monocytes to the site of tumor cell injection and, once there, secreted JE/MCP-1 protein induced monocyte tumoricidal activity. To test this hypothesis, JE/MCP-1-expressing cells were mixed with 10^7 0A-2 cells, a number of cells that reproducibly led to tumor formation when injected alone (Table 1). Coinjection of murine JE/MCP-1-expressing cells (10A-10) with 0A-2 cells completely suppressed tumor formation in two animals (Fig. 2A). (This finding was replicated five additional times.) Coinjection of high-level human JE/MCP-1-expressing cells (hJEC-100) with 0A-2 cells also completely suppressed tumor formation. Coinjection of low-level human JE/MCP-1-expressing cells (hJEC-10) suppressed tumor formation for 8 to 10 days, after which tumors appeared. Presumably, hJEC-10 cells exerted a suppressive effect transiently until the proliferating 0A-2 cell mass reached a size that enabled it to escape the effect.

JE/MCP-1 expressors and nonexpressors were also coinjected with HeLa cells to test whether JE/MCP-1 expression could suppress tumor formation by another cell type. Figure 2B shows that 10A-10 cells completely suppressed tumor formation by HeLa cells. As described above, coinjection with hJEC-10 cells delayed tumor formation. In a separate experiment, four of four animals injected with 10^7 HeLa cells formed tumors while only one of four animals injected with 10^7 HeLa cells plus 10^7 hJEC-100 cells formed tumors. Mice that displayed no tumor growth after receiving HeLa plus hJEC-100 cells were then injected with 10^7 HeLa cells alone. These mice developed large tumors within 14 days, indicating that prior suppression of HeLa cell tumor growth in the presence of JE/MCP-1 does not render mice immune to rechallenge with HeLa cells.

Histologic examination of the tumors arising from coinjected HeLa and 0A-2 cells revealed a mixture of epithelioid HeLa cells and spindle-shaped CHO cells. Examination of the tumors formed in animals that received HeLa and hJEC-10 cells also demonstrated a mixture of HeLa and CHO cells. The presence of some hJEC-10 cells in these tumors was confirmed by Northern (RNA) blot analysis in which expression of human *JE* mRNA was detected (data not shown). After reaching a certain size, these tumors may overwhelm the host response elicited by the low levels of human JE/MCP-1 secreted by hJEC-10 cells. However, tumor growth still requires the presence of a malignant JE/MCP-1-nonexpressing cell line, since hJEC-10 cells injected by themselves cannot form tumors (Table 1). This also suggests that the intrinsic growth properties of CHO cells were not altered by JE/MCP-1 expression and that human JE/MCP-1 expressors will proliferate in vivo if protected by an enlarging mass of malignant cells.

MCP-1/JE expression elicits a monocytic infiltrate. Figures 3 and 4 show the results of a histological examination of the sites of CHO cell inoculation 24 h after the cells were injected. JE/MCP-1 nonexpressors (darkly stained, large cells indicated by arrows in Fig. 3A) induced the appearance in the underlying connective tissue of only a few cells, most of which were neutrophils. In contrast, CHO cells expressing murine JE/MCP-1 (Fig. 3B, arrows) elicited an abundant cellular infiltrate. At higher power (Fig. 4A), it is apparent that this infiltrate consisted primarily of monocytes with a reproducibly significant proportion of eosinophils (arrows), usually 10 to 20%. As might be expected, low-level human JE/MCP-1-expressing cells (hJEC-10) induced a qualitatively similar infiltrate (Fig. 4B) that was intermediate in intensity between the nonexpressors and the murine JE/MCP-1 expressors (compare Fig. 3C with A and B).

DISCUSSION

We have demonstrated that expression of the *JE* gene in malignant CHO cells suppresses their ability to form tumors in nude mice. This apparent phenotypic reversion requires

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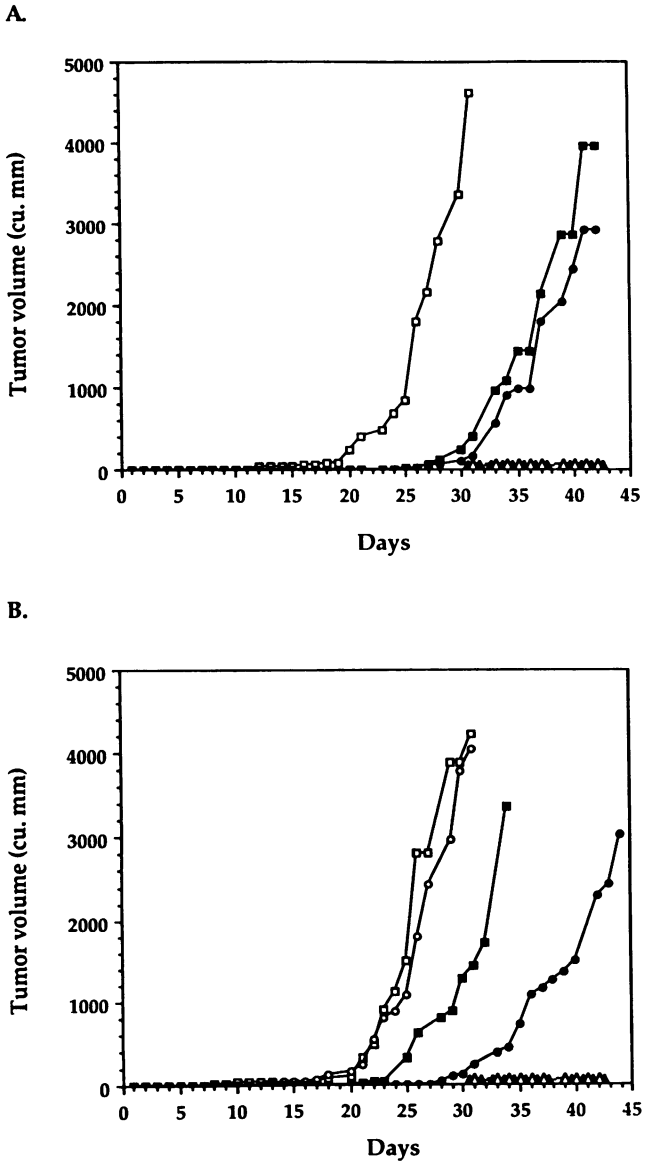

FIG. 2. Growth rates of tumors in nude mice. Four- to six-week-old Swiss *nu/nu* mice were injected subcutaneously with mixtures of CHO and HeLa cells. Tumor volumes were derived by multiplying three perpendicular diameters. Each symbol represents a different mouse. (A) CHO cells only. Symbols: □, 10^7 0A-2 cells; ■ and ●, 10^7 0A-2 cells plus 10^7 hJEC-10 cells; ▲ and △, 10^7 0A-2 cells and 10^7 10A-10 cells (identical results were obtained with 10^7 0A-2 cells and 10^7 hJEC-100 cells). (B) CHO and HeLa cells. Symbols: □ and ○, 10^5 HeLa cells and 10^7 0A-2 cells; ■ and ●, 10^5 HeLa cells and 10^7 hJEC-10 cells; ▲ and △, 10^5 HeLa cells and 10^7 10A-10 cells (identical results were obtained with 10^7 HeLa cells and 10^7 hJEC-100 cells in three of four animals [see text]).

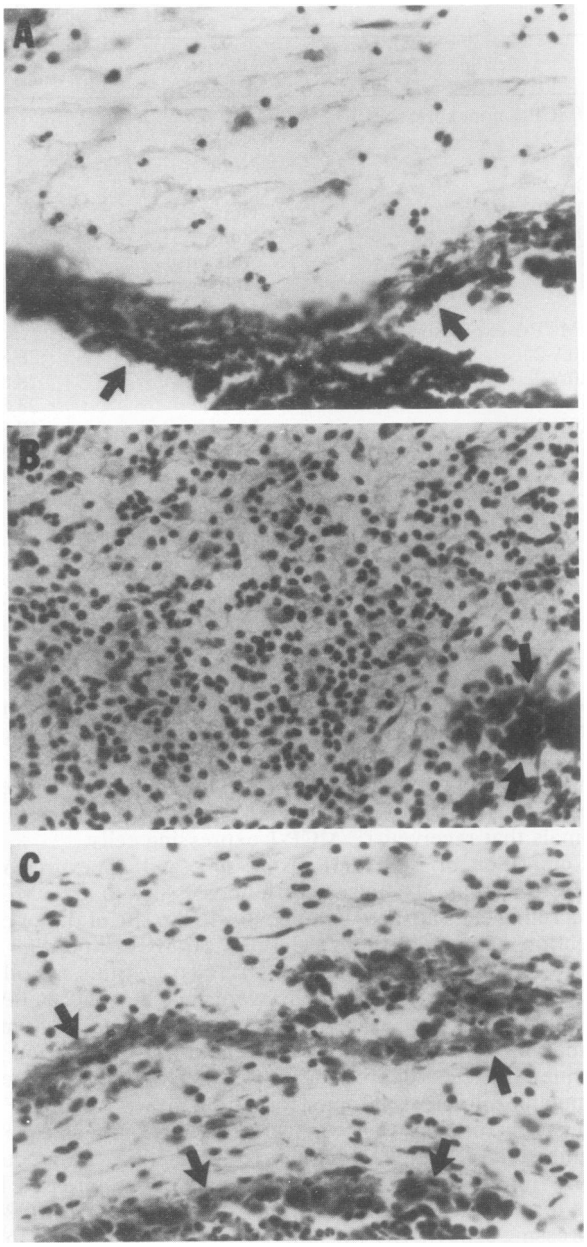

FIG. 3. Cellular infiltrate at the site of CHO cell injection. Male Swiss *nu/nu* mice were injected subcutaneously with 10^7 0A-10, 10A-10, or hJEC-10 cells. The mice were sacrificed 24 h later, and the injection sites were processed for histological examination. All sections were stained with hematoxylin and eosin. (A) 0A-10. The densely packed, hyperchromic cells across the bottom of the field are CHO cells (arrows). A mixture of neutrophils and mononuclear cells is present in loose connective tissue. (B) 10A-10. CHO cells are visible in the lower right corner of the field (arrow). A predominantly monocytic infiltrate with occasional eosinophils is present (see Fig. 4A). (C) hJEC-10. CHO cells are apparent at the bottom and as a band through the center of the field (arrows). An infiltrate similar in composition to that in panel B, but less intense, is apparent (see Fig. 4B). Magnification, ×400.

interaction with host factors in vivo, since expression of JE/MCP-1 does not alter the transformed character of these cells in vitro. Furthermore, we have shown that JE/MCP-1-expressing cells exert their effect in *trans* by the ability to suppress tumor formation when coinjected with JE/MCP-1-nonexpressing CHO cells or HeLa cells. These observations are consistent with the in vitro monocyte chemoattractant and stimulant properties of the product of the *JE* gene (16, 19a, 27, 32). While we demonstrated a predominantly monocytic infiltrate at the site of tumor cell injection, our experiments do not prove that monocytes mediate tumor growth suppression in this system. The effect is probably not

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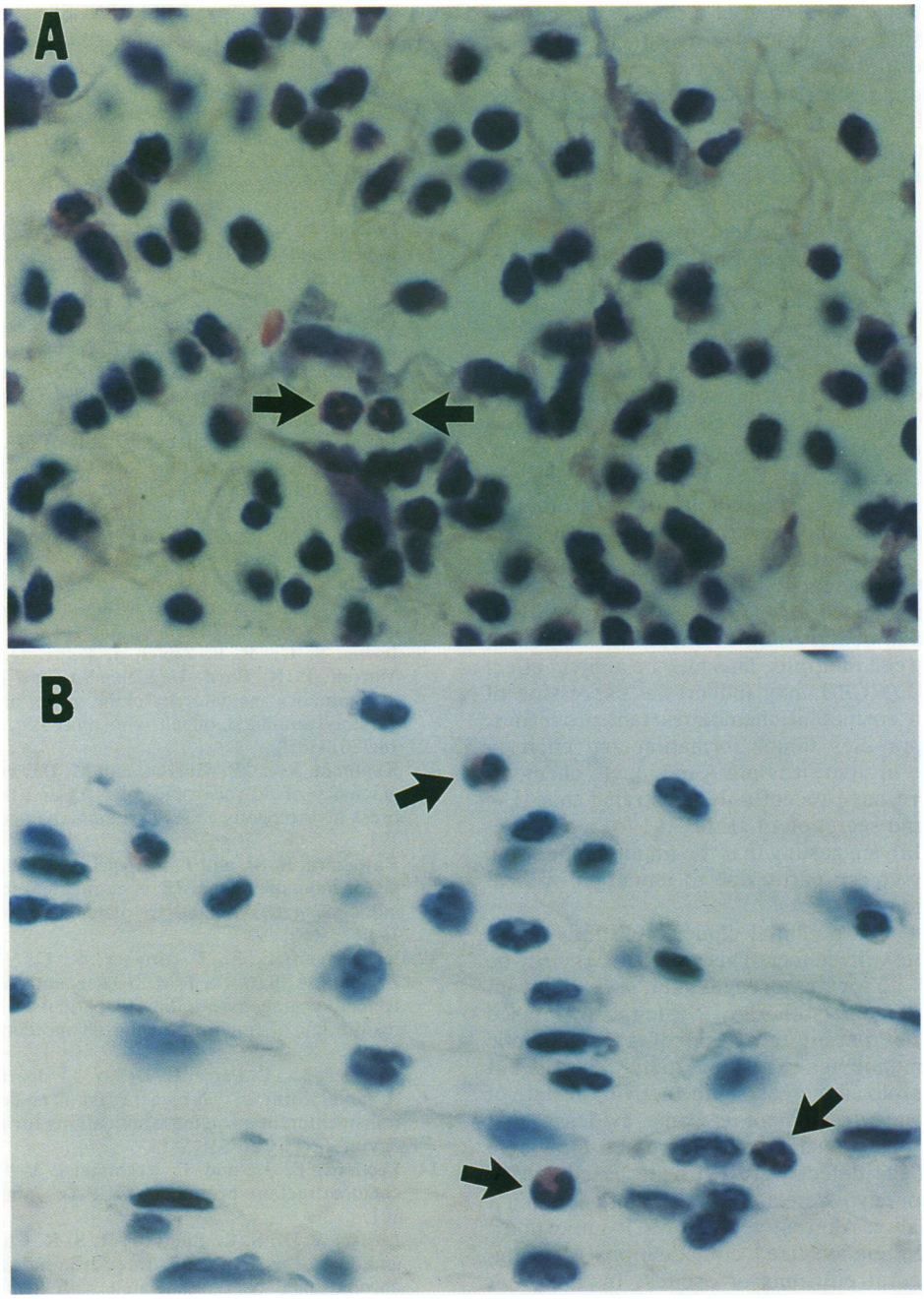

FIG. 4. (A) Cellular infiltrate elicited by 10A-10 at higher magnification. A pair of eosinophils can be seen (arrows). (B) Cellular infiltrate elicited by hJEC-10 at higher magnification. Numerous eosinophils are present (some indicated by arrows). Magnification, ×800.

mediated by T lymphocytes in these young athymic mice and is not likely to be mediated by natural killer cells, since we have shown that purified recombinant JE/MCP-1 protein has no stimulatory effect on natural killer cells in vitro (19a). The in vitro effects of JE/MCP-1 on monocytes and its in vivo elicitation of a monocytic infiltrate suggest that monocytes are the most likely mediators of the suppressive effects of JE/MCP-1.

We also have not demonstrated directly that JE/MCP-1 protein expression mediates tumor suppression. Our anti-JE/MCP-1 serum had negligible neutralizing capacity in vitro, and we were unable to use this serum to block the JE/MCP-1 effect in vivo. Instead, we tried to control for as many variables as possible by using several independently derived cell lines that were otherwise isogenic except for JE/MCP-1 expression. In addition, the low-level human JE/MCP-1-expressing cells (hJEC-10) were less potent tumor suppressors in coinjection experiments than were the high-level human JE/MCP-1 expressors (hJEC-100). This dose response suggests that the tumor-suppressive effect is mediated by the JE/MCP-1 protein.

If monocytes are responsible for tumor suppression in this system, there are several possible mechanisms whereby JE/MCP-1-activated monocytes might exert their effects.

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JE/MCP-1 could induce expression of a soluble mediator of tumor cell lysis, such as tumor necrosis factor (17, 25). Alternatively, tumor necrosis factor could be expressed and displayed in an active form on the surfaces of activated monocytes (13). It is possible, however, that while JE/MCP-1 is necessary for the tumor suppression effect we observed, it is not sufficient. JE/MCP-1 could play an accessory role in a primary immunologic response to transplanted tumor cells, although the inability of nude mice to reject HeLa cells on rechallenge argues against this possibility. These hypotheses are now being explored in vitro and in syngeneic animal systems.

Our findings are similar to those of Tepper et al., who investigated murine interleukin 4 (IL-4) (24). As in our experiments, expression of IL-4 in several tumor cell lines inhibited the in vivo growth of expressing lines, as well as that of coinjected nonexpressing tumor lines. In contrast to their observations, none of our JE/MCP-1-expressing cell lines formed tumors of any size, precluding demonstration of a monocytic infiltrate into the tumors themselves. This may attest either to the greater susceptibility of CHO and HeLa cells to monocyte tumoricidal activity or to a more effective host cell infiltrate induced by JE/MCP-1 than by IL-4. Interestingly, we also observed an eosinophilic component to the inflammatory cell infiltrate. This may be a direct effect of JE/MCP-1, or JE/MCP-1 may induce the expression of another factor with eosinophil chemoattractant properties. Although IL-4 suppresses tumor formation and elicits a monocytic infiltrate in vivo, it is not a monocyte chemoattractant in vitro. We have recently demonstrated that IL-4 induces synthesis and secretion of JE/MCP-1 from endothelial cells in vitro (21a), suggesting that the tumor suppressive effects of IL-4 in vivo are partly due to induction of local JE/MCP-1 expression.

Our results point to a potential clinical role for infused JE/MCP-1 in patients with cancer. This would necessarily be limited to patients with low tumor loads, i.e., as an adjuvant to surgery or cytotoxic chemotherapy. Systemically infused JE/MCP-1 would not permit formation of a chemotactic gradient to attract monocytes to residual tumor deposits but might lead to a generalized increase in the activation state of an individual's monocytes. There is some evidence that patients harboring malignancies have depressed monocyte function (2, 12). If the defect were cytokine based, rather than an inherent monocyte defect, JE/MCP-1 infusion might correct the abnormality. Alternatively, JE/MCP-1 may find a use in the treatment of localized complications of malignancy, such as pleural effusions or ascites. Instilling JE/MCP-1 into the involved anatomic space might lead to local monocyte accumulation and activation. Similarly, engineering tumor-infiltrating lymphocytes (TIL cells) to express JE/MCP-1 might provide synergistic local tumor cell killing.

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