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Advances in Brief

Human Lactoferrin Inhibits Growth of Solid Tumors and Development of Experimental Metastases in Mice[1]

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Abstract

The antitumor effects of the multifunctional iron-binding glycoprotein, lactoferrin (Lf), were investigated. Lf inhibited growth in mice of transplantable solid tumors induced by v-*ras* transformed fibroblasts and a methylcholanthrene-induced fibrosarcoma. Lf also substantially reduced lung colonization (experimental metastasis) by B16-F10 melanoma cells in syngeneic mice. Iron-saturated and apo-Lf exhibited comparable levels of tumor inhibition and antimetastatic activity. Transferrin, a related iron-binding protein, had no effect on lung colonization. In the B16-F10 system, elimination of natural killer cell activity by pretreatment of mice with anti-asialo G_{M1} antibody abrogated the effects of Lf, whereas inhibition of macrophage function with silica did not. The results demonstrate a novel activity for Lf and suggest a potentially important role for this molecule in the primary defense against tumorigenesis.

Introduction

The principal function of Lf[3], the most avid iron-binding member of the Tf family of proteins, is considered to be the prevention of microbial infection through sequestration of iron required for microbial growth (1). However, a wide array of additional functions have been attributed to Lf, many related to host primary defense mechanisms. Thus, Lf activates NK cells (2, 3), induces colony stimulating activity (4), activates PMN (5), regulates granulopoiesis (6), enhances antibody-dependent cell cytotoxicity activity (7), stimulates LAK cell activity (2), and potentiates macrophage cytotoxicity (5) among other effects. Interestingly, a number of these activities appear to be independent of the iron-binding function of Lf (7, 8). Despite the numerous and varied functions attributed to Lf, the complete spectrum and true role of its activity in primary *in vivo* resistance against infectious or invasive processes have not yet been elucidated. Moreover, the mechanisms by which Lf may mediate these varied effects have not been determined. We have examined the antitumor effects of Lf using established tumor models in mice. We found that Lf reduced solid tumor growth and strongly inhibited experimental metastasis (lung colonization). The effects of Lf on experimental metastasis appeared to be mediated through NK cells and were independent of iron saturation. This is the first demonstration to our knowledge of an antitumor effect attributed to Lf. The results point to a potential role for this multifunctional primary defense protein in protection from tumor development or progression and may offer novel approaches to therapy.

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[3] The abbreviations used are: Lf, lactoferrin; Tf, transferrin; NK, natural killer; PMN, polymorphonuclear leukocytes; LAK, lymphokine-activated killer; PBS, phosphate-buffered saline; LPS, lipopolysaccharide.

Materials and Methods

Tumor Models. All cell lines were maintained as monolayers in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum at 37°C in a humidified incubator with 5% CO_2 in air. Cells were trypsinized and suspended in Dulbecco's PBS in preparation for injection. Syngeneic C57BL/6 mice were inoculated i.v. with 7.5×10^4 B16-F10 melanoma cells. Animals were sacrificed 21 days after injection, and the lungs were excised and fixed in 10% formalin. Metastatic lesions were counted with the aid of a stereo microscope. MCA4-P5 (a cloned methylcholanthrene-induced fibrosarcoma developed in our laboratory) and NIH 3T3 rp1A (a cloned v-*ras*-transformed NIH 3T3 cell line, kindly provided by Dr. Ruth Muschel, University of Pennsylvania, Philadelphia, PA) were injected s.c. into syngeneic NIH/PLCR inbred mice. The tumor cross-sectional diameters were measured using calipers and were used to calculate volumes with the formula $(L \times W^2)/2$.

Animals. Female C57BL/6 mice, 14–18 g on receipt, were obtained from Taconic Farms (Germantown, NY). Animals were observed for 2 weeks prior to experimentation in order to assure freedom from disease or adventitious agents. NIH/PLCR mice were produced in our facility by brother-sister mating and were free of common murine infectious agents. All animals were maintained in accord with accepted practices with food and water provided *ad libitum*; all experimental protocols were approved by the Institutional Animal Care and Use Committee.

Reagents. Dulbecco's minimum essential medium and PBS were purchased from Gibco (Grand Island, NY). Fetal calf serum was purchased from HyClone (Logan, UT) and was heat inactivated at 56°C for 30 min prior to use. Human Lf, human apo-Tf, and human albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Solutions of each were prepared in PBS. Rabbit anti-asialo G_{M1} was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Mice were injected i.p. with 0.5 ml of a 1:20 dilution of the stock prepared according to the manufacturer's directions. Silica (Min-U-Sil; particle size <5 μm) was purchased from Whitaker, Clark, and Daniels Minerals, Inc. (Detroit, MI) and was suspended in PBS by sonication. Mice were inoculated i.v. with 2 mg silica. *Limulus* amebocyte lysate test kit was purchased from Whitaker (Walkersville, MD) and used according to the manufacturers' directions to test for LPS.

Statistical Analysis. The tumor growth rates were compared by repeated measures of two-way analysis of variance with multiple comparisons made using Bonferroni's *t* test. Lung colonization data were analysed by the Mann-Whitney *U* test.

Results

Two transplantable solid tumor models, syngeneic to inbred NIH/PLCR mice, were used to test the antitumor effects of Lf: MCA4-P5, a cloned methylcholanthrene-induced fibrocarcinoma; and NIH 3T3 rp1A, a highly malignant v-*ras*-transformed NIH 3T3 cell line. Growth of NIH 3T3 rp1A-induced tumors was significantly reduced by a single i.p. injection of 1 mg of Lf 24 h after tumor cell inoculation. Growth of MCA4-P5 tumors also showed a significant reduction following treatment with Lf. Importantly, the growth of MCA4-P5 tumor was reduced equally by either apo-Lf or iron-saturated Lf. Typical results are shown in Fig. 1.

These results were extended to the B16-F10 experimental metastasis model in C57BL/6 mice. As shown in Table 1, a single i.p. injection of 1 mg of Lf 24 h prior to i.v. injection of tumor cells

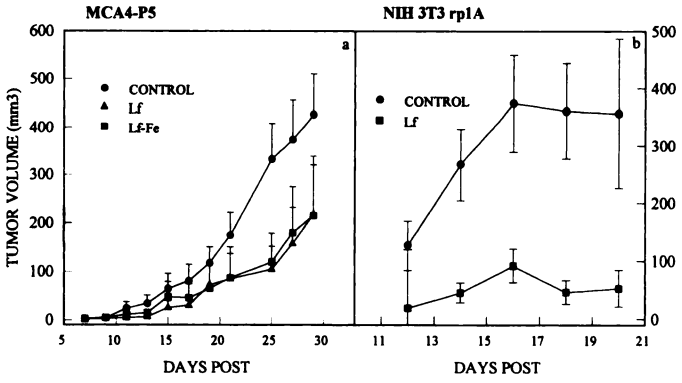

Fig. 1. The effects of Lf and iron-saturated Lf on the growth of solid tumors. *a, MCA4-P5* [Lf and iron-saturated Lf significantly different than controls ($P < 0.001$); analysis of variance corrected by Bonferroni's *t* test]; *b, NIH 3T3 rp1A* [Lf treatment different than control ($P < 0.001$); Bonferroni's *t* test]. *Bars,* SD.

Table 1 *Effect of Lf on B16-F10 melanoma lung colonization*[a]

Treatment	Incidence of lung lesions	Median no. of lesions[b]	No. of individual lesions
Control	8/9	41	0, 28, 34, 40, 41, 42, 45, 51, 59
1 mg Lf	7/8	9[c]	0, 1, 2, 9, 10, 15, 16, 17
100 μg Lf	7/8	18	0, 6, 8, 16, 18, 19, 19, 35
1 mg Lf-Fe	6/8	13[c]	0, 0, 1, 12, 14, 15, 15, 34
1 mg Lf 24 h post	8/9	3[d]	0, 1, 1, 2, 3, 4, 6, 11, 31
100 μg Lf 3 × post	9/9	11[c]	5, 6, 7, 9, 13, 22, 24, 26

[a] B16-F10 melanoma cells (7.5×10^4) were injected i.v. into each mouse. On day 21 postinjection, the animals were sacrificed, and lungs were placed in 10% formaldehyde.
[b] Visible lesions under stereomicroscope (× 10).
[c] Significantly different from control ($P < 0.02$; Mann-Whitney *U* test).
[d] Significantly different from control ($P < 0.001$; Mann-Whitney *U* test).

substantially reduced lung colony formation. Lf administered 24 h after cells were inoculated was also effective. A dose of 100 μg of Lf given prior to tumor cells were inoculated or three daily doses of 100 μg each given after tumor cells were inoculated significantly reduced colony formation, although not to the extent of that achieved with 1 mg. Treatment with iron-saturated Lf gave the same result as treatment with apo-Lf.

The effects of Lf on experimental metastasis were not seen with the closely related iron-binding protein Tf or with a non-iron-binding protein, albumin, as shown in Table 2. The effects of Lf did not appear to be due to contamination with LPS, which is known to bind to Lf; the Lf preparations used had just detectable levels of LPS using the Limulus lysate assay (sensitivity > 0.06 endotoxin units/ml of endotoxin), well below the levels of LPS known to reduce lung colonization or to activate NK tumoricidal activity (9–11). Also, the control Tf and albumin preparations, which did not reduce lung colonization, had comparable levels of LPS. Lf had no significant effect on *in vitro* growth of B16-F10 cells or the other cell lines used (data not shown).

To elucidate the possible mechanism of Lf action in reducing experimental metastasis, animals were treated with silica or anti-asialo G_{M1} to eliminate macrophages and NK cell activity, respectively, prior to treatment with Lf and then they were inoculated with tumor cells. These targets were chosen because both cell types are known to be involved in resistance to lung colonization, as demonstrated using similar protocols for their elimination (9, 12), and both are known to be influenced by Lf (2, 3, 5). As shown in Table 3, pretreatment with anti-asialo G_{M1} markedly increased lung colonization by B16-F10 cells, whereas silica had a slight effect. Similar results have been reported with these reagents in this system (12). Lf treatment was as effective in reducing lung colonies in silica-treated animals as in controls. In contrast, the effects of Lf were totally abrogated in animals treated with anti-asialo G_{M1}, suggesting the involvement of NK cells in Lf antimetastatic activity.

Discussion

Lf is a multifunctional iron-binding protein thought to be responsible for host primary defense against microbial infection through very avid binding of iron required for microbial growth (1). However, many other functions have been attributed to Lf, suggesting a much broader role in host resistance to infection and other invasive processes. Here we show that Lf also inhibits solid tumor growth and experimental metastasis in mice, activities which are independent of the characteristic iron-binding function of Lf.

Although antitumor effects of Lf have not been reported previously, PMN leukocytes, the principal source of circulating Lf, have been shown to be involved in tumor rejection (13–15). For example, selective elimination of PMN with antibody RP-3 eliminated tumor-associated antigen transplantation resistance to syngeneic tumors in rats (14). PMN alone, and more so in the presence of tumor necrosis factor-α, suppressed tumor cell proliferation *in vitro* (15). The relationship between these effects of PMN and Lf *in vivo* is not known.

Mediation of the antitumor activity of Lf through NK cells is in accord with previous findings on the *in vitro* and *in vivo* stimulation of NK and LAK cell activity by Lf (2, 16) and the well-known antitumor effects of NK cells (9). The mechanism of Lf stimulation of NK cell activity is not known; Lf could act directly or through an influence on elaboration of cytokines. The latter is a well-described function of Lf, although for the most part, Lf is thought to decrease

Table 2 *Effects of Lf, Tf, and albumin in B16-F10 lung colonization*[a]

Treatment	Incidence of lung lesions	Median no. of lesions[b]	No. of individual lesions
Control	11/12	35	0, 8, 18, 22, 30, 32, 38, 38, 42, 48, 56, 85
1 mg Lf	5/10	0.5[c]	0, 0, 0, 0, 0, 1, 2, 5, 12, 13
1 mg Tf	9/10	32	0, 4, 22, 26, 31, 33, 33, 34, 34, 69
1 mg albumin	10/10	38.5	3, 11, 22, 29, 38, 39, 42, 45, 51, 55
1 mg Lf post	5/10	1.5[c]	0, 0, 0, 0, 0, 3, 5, 8, 14, 21
1 mg Tf post	9/10	39.5	0, 23, 25, 29, 36, 43, 56, 74, 76, 78

[a] B16-F10 melanoma cells (7.5×10^4) were injected i.v. into each mouse. On day 21 postinjection, the animals were sacrificed, and the lungs were placed in 10% formaldehyde.
[b] Visible lesions under stereoscope (× 10).
[c] Significantly different than control ($P < 0.001$; Mann-Whitney *U* test).

Table 3 *Lf treatment of B16-F10 melanoma: effects of elimination of NK cells and macrophages*[a]

Treatment	Incidence of lung lesions	Median no. of lesions[b]	No. of individual lesions
Control	9/9	11	2, 8, 9, 10, 11, 14, 14, 18, 27
1 mg LF	3/7	0[c]	0, 0, 0, 0, 1, 1, 35
1 mg LF-Fe	3/7	0[c]	0, 0, 0, 0, 1, 1, 18
Anti-asialo G_{M1}	7/7	85+[d]	49, 85, 85, 85, 85, 85, 85
Anti-asialo G_{M1} + 1 mg LF	8/8	85+	13, 24, 85, 85, 85, 85, 85, 85
Silica	5/6	16	0, 11, 15, 16, 38, 39
Silica + 1 mg LF	3/7	0[c]	0, 0, 0, 0, 2, 3, 9

[a] B16-F10 melanoma cells (7.5×10^4) were injected i.v. into each mouse. On day 21 postinjection, the animals were sacrificed, and the lungs were placed in 10% formaldehyde.
[b] Visible lesions under stereoscope (× 10).
[c] Significantly different from control ($P < 0.001$; Mann-Whitney *U* test).
[d] Eighty-five or more lesions, *i.e.*, too numerous to count.

expression of cytokines such as interleukin 1, interleukin 2, and tumor necrosis factor-α (2).

In addition to its antimicrobial effects, Lf has been shown to reduce infection of mice with the Friend murine leukemia virus complex (16). Lf alone, administered at the same time as the virus but not afterwards, inhibited viral infection, possibly due to suppression of replication of hematopoietic stem cells that are the targets of the virus. Lf in combination with γ-interferon was highly effective in the treatment of established infection, which was suggested to be due to restoration of diminished NK function in infected animals.

The effects of Lf on tumor growth might have been due to the removal of iron required for tumor cell proliferation by analogy to the mechanism of Lf-mediated resistance to microbial infection. Iron chelation is known to reduce tumor cell growth (17). However, the fact that iron-saturated Lf is as active in antitumor effects as apo-Lf argues strongly against this mechanism. It remains possible that iron-saturated Lf could dissociate *in vivo* and still mediate its effects locally through iron binding, although the very high association constant for iron and Lf (and the substantial iron stores available in the body) makes this an unlikely prospect. Additionally, *in vitro* activation of NK and LAK by Lf is not inhibited by the iron chelator deferoxamine.

There may be potential for exploiting this novel antitumor activity of Lf for therapeutic purposes. The high concentrations of Lf present in epithelial secretions and its general lack of toxicity would make it an attractive candidate agent. Substantial effects on metastasis would be an obvious benefit. Action through NK cells suggests that combination therapies with other immunomodulators could be useful. Such an approach was effective in suppressing Friend virus infection in mice, as noted above (16). Use of exogenous Lf to combat microbial infections has been suggested, although this might be limited by the fact that some microbes produce siderophores that are able to extract the tightly bound iron from Lf (17). Such limitations should not be factors in Lf antitumor activity.

Further studies are under way to elucidate more fully the mechanism of Lf effects on tumorigenesis, to better define the role of NK and other cells in the process, and to examine the potential for use of Lf as a therapeutic agent. Obviously, effects of Lf on long-term, established primary tumors and on spontaneous metastasis must be examined. The normal role for endogenous Lf in resistance to tumor development is also of interest and is presently under study in our laboratory.

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