Prevention of Adenocarcinoma Colon 26-induced Cachexia by Interleukin 10 Gene Transfer

Fujio Fujiki, Naofumi Mukaida, Kunitaka Hirose, Hiroshi Ishida, Akihisa Harada, Shinisuke Ohno, Horst Bluetthmann, Masanobu Kawakami, Mariko Akiyama, Saburo Sone, and Kouji Matsushima

Departments of Pharmacology [F. F., N. M., M. A., K. M.] and Molecular Immunology [S. O.], Cancer Research Institute, Department of Hygiene, School of Medicine [A. H.], Kanazawa University, 1-3-1 Takara-machi, Kanazawa 920, Japan; Third Department of Internal Medicine, School of Medicine, University of Tokushima, 2-3-1, Kuramoto-cho, Tokushima 770, Japan [F. F., S. S.]; Department of Internal Medicine, Clinical Research Center, National UTano Hospital, 8 Onodoyama-cho Narutaki, Ukyo-ku, Kyoto 615, Japan [H. I.; Biomedical Research Institute, Kureha Chemical Industry, 3-26-2 Hyakunin-cho, Shinjuku-ku, Tokyo 169, Japan [K.H.]; Department of Biology, Pharmaceutical Research Gene Technology, Hoffmann-La Roche Ltd., Basel, Switzerland [H. B.]; Department of Integrated Medicine, Omiya Medical Center, Jichi Medical School, Omiya, Saitama 330, Japan [M. K.]; and Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan [K. M.]

ABSTRACT

A s.c. injection of a mouse colon adenocarcinoma cell line, colon 26 clone 20, induced cachexia, as evidenced by progressive weight loss and severe hypoglycemia. Several lines of evidence indicate that a pro-inflammatory cytokine, interleukin 6 (IL-6), plays a major role, albeit partially, in the establishment of cachexia in this model. Because IL-10 can potently inhibit the production of pro-inflammatory cytokines, including IL-6, we evaluated the effects of IL-10 gene transfer on the establishment of cachexia. IL-6 transcript was detected at tumor sites of mice inoculated with parental or control vector transfected cells, and serum IL-6 levels were markedly increased in these mice. The injection of parental cells into IL-6-deficient mice induced cachexia with elevated serum IL-6 levels comparable to wild-type mice, indicating that tumor cells are a major source of IL-6. The inoculation of IL-10-transfected cells kept IL-10 mRNA expression at tumor sites and induced the elevation in serum IL-10 levels without affecting the growth rates of colon 26 cells both in vitro and in vivo. However, the implantation with IL-10-transfected cells reduced the expression of IL-6 mRNA at the tumor sites and the elevation in serum IL-6 levels. Concomitantly, mice inoculated with IL-10-transfected cells did not exhibit progressive weight loss, a reduction in food intake, or severe hypoglycemia, which was observed in mice inoculated with parental or control vector-transfected cells. Collectively, these results suggest that IL-10 gene transfer prevented the occurrence of cachexia with a concomitant inhibition of IL-6 production at the tumor sites.

INTRODUCTION

Cachexia, characterized by progressive weight loss, anemia, and asthenia (1), remains an intractable condition in cancer patients because it cannot be reduced by a simple compensation for the negative caloric balance through total parenteral nutrition (2). Moreover, patients with cachexia have a poor prognosis due to a reduced response to chemotherapy (3). Hence, an appropriate treatment modality against cachexia should be developed to improve the prognosis as well as the quality of life of the patients. Cachexia occurs in patients even when the tumor burden is less than 1% of body weight (4). Thus, it is desirable that a progressive body weight loss develop with a relatively small tumor burden, even in an animal model of cancer cachexia. s.c. injection of a murine adenocarcinoma cell line, colon 26, causes cachexia even when the tumor remains a relatively small burden, implying that this cell line is useful for elucidating the mechanism of cachexia (5, 6). In this model, we reported previously that endogenously produced IL-6 was mainly involved in inducing cachexia (7).

IL-10, originally identified as a cytokine synthesis inhibitory factor (8), reduces the antigen-presenting capacities of monocytes, thereby inhibiting the T cell-derived cytokines such as IL-2 and IFN-γ (9–11). Moreover, it turned out that IL-10 potently inhibits the production of pro-inflammatory cytokines including IL-1, IL-6, IL-8, and TNF-α by monocytes (10, 12, 13), thereby preventing endotoxin-induced acute lethality (14). Hence, we have evaluated here the effects of mouse IL-10 gene transduction into colon 26 cells on the course of cachexia.

MATERIALS AND METHODS

Mice. Pathogen-free, 8-9-week old female BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan). To get IL-6-deficient mice, animals heterozygous for a germ-line null mutation of the IL-6 gene (15), were back-crossed to BALB/c mice for six generations. Resultant mice were interbred to yield homozygous IL-6-deficient mice. Homozygotes were further interbred to generate sufficient numbers of mice for the experiments. Mice weighing between 19 and 23 g were used for the experiments, complying with the standards set out in the “Guidelines for the Care and Use of Laboratory Animals” on the Takara-machi campus of Kanazawa University.

Construction of Mouse IL-10 Expression Plasmid. The cDNA encoding the full-length mouse IL-10 cDNA was isolated from total RNA extracted from LPS-stimulated mouse splenocytes by reverse transcription-PCR using a set of primers based on the reported nucleotide sequence of mouse IL-10 (8). The fragment was subcloned into a eukaryotic expression vector, pH3Apr-1, which contains a neomycin-resistant gene and can express the cloned foreign gene under the control of human β-actin promoter (16). The nucleotide sequence of the obtained plasmid, pH3Apr-IL-10, was determined by Sanger’s method before transfection into cells (17).

Transfection of Colon 26 Cells. Clone 20, derived from a murine colon 26 adenocarcinoma cell line, was cultured in vitro with the complete medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mm glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin, as described previously (7). Clone 20 cells (100,000) were transfected with 2 μg of either pH3Apr-1 or pH3Apr-IL-10 plasmid using LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. To obtain stably transfected clones, transfected cells were grown in the complete medium containing G418 (Life Technologies, Inc.) at a final concentration of 600 μg/ml for 14 days, and resistant clones were propagated separately. The capacity of each transfected clone to produce mouse IL-10 was confirmed by determining IL-10 concentrations in the supernatants of 1 × 10⁶ cells cultured for 48 h in 1 ml of medium by a specific ELISA. In some experiments, the cells were incubated with or without the indicated concentrations of recombinant human IL-1α (a kind gift from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan; specific activity, 1 × 10⁷ units/mg) to extract total RNAs.

Inoculation of Tumor. Cells in a subconfluence were treated with trypsin and resuspended at 2.5 × 10⁶ cells/ml in PBS (–). Forty μl of the cell suspensions were inoculated s.c. into the right rear footpad of each mouse. The thickness of the
footpad and the body weight were measured every other day, whereas the amount of food intake was determined every day. Sera were obtained from blood collected from the axillary artery before sacrifice and were kept at –80°C before the measurements. Total RNAs were extracted from tumors in the footpad and spleens at 15 days after the inoculation of the tumor.

**Northern Blot Analysis.** The cDNA fragments encoding the whole coding region of mouse IL-10 (8), IL-6, IL-1β, TNF-α, IFN-γ, transforming growth factor β were obtained and confirmed as described (7). Twenty μg of extracted total RNAs were loaded onto each lane on a 1% agarose gel containing formaldehyde. Northern blot analysis was performed using the above-mentioned cDNA fragments as a probe as described previously (7). The probed nylon membranes were analyzed using a BAS1000 Bioimage analyzer (Fuji Film, Tokyo, Japan). Hybridization with mouse 18S rRNA confirmed that equal amounts of RNA were loaded for each blotting analysis.

**Determination of Serum Cytokine Levels.** Serum levels of IL-10 and IL-6 were measured by using a mouse IL-10 and a mouse IL-6 ELISA Kit (Biosource), respectively.

**Determination of Blood Glucose Levels.** Blood glucose levels were determined using Fuji Dry Chem FDC5500 (Fuji Film) according to the manufacturer’s instructions.

**Statistical Analysis.** The means and SDs were calculated on all of the parameters determined in this study. Statistical analyses was performed using two-way ANOVA, followed by a multiple comparison according to the Turkey-Kramer procedure; P < 0.05 was accepted as statistically significant. A generalized Wilcoxon test of Kaplan-Meier curves was used to evaluate the statistical significance of survival rates.

**RESULTS**

**In Vitro Characteristics of Colon 26 Clone 20 Cells Transfected with Mouse IL-10 Gene.** The transfection of colon 26 clone 20 cells with mouse IL-10 gene gave rise to two constitutively IL-10-producing clones, designated clone 20/IL-10-1 and clone 20/IL-10-2. Northern blot analysis revealed that both clones constitutively expressed much higher levels of IL-10 mRNA than parental cells, which barely expressed IL-10 mRNA (data not shown). Moreover, both clones produced mouse IL-10 protein (clone 20/IL-10-1, 20.4 ng/ml/48 h; clone 20/IL-10-2, 77.6 ng/ml/48 h), whereas neither parental cells nor cells transfected with a control vector (clone 20/vector-1) produced a detectable level of IL-10. The clones transfected with IL-10 gene showed similar growth rates in vitro as the parental cells and clone 20/vector-1 (data not shown).

Moreover, IL-1 induced IL-10 transfecants to express IL-6 mRNA and to produce IL-6 protein to a similar level as parental clone 20 or clone 20/vector-1 cells (Fig. 1). These results suggested that the transduction of mouse IL-10 gene into these cells had negligible effects on its in vitro growth and IL-6-producing capacity.

**The Effects of IL-10 Gene Transduction on Colon 26 Clone 20-induced Cachexia.** IL-10 transcript was detected at the tumor sites of mice inoculated with IL-10 transfecants but neither parental nor cells transfected with a control vector (Fig. 2). Moreover, IL-10 transcript was not detected at spleens from mice inoculated, even with IL-10 transfecants. These results suggest that IL-10 was locally produced by the implanted cells transfected with IL-10 gene. Furthermore, serum IL-10 levels in mice implanted with clone 20/IL-10-1 or clone 20/IL-10-2 were significantly higher than those with parental cells or clone 20/vector-1 (Fig. 3). However, no significant difference was observed in the tumor growth rates among mice inoculated with parental cells, clone 20/vector-1, clone 20/IL-10-1, and clone 20/IL-10-2 until 15 days after the inoculation (Fig. 4), implying that IL-10 gene transduction had negligible effects on tumor growth, even in vivo. Consistent with the previous report (18), mice implanted with a parental cell exhibited a progressive weight loss and a reduction in food intake, starting at 12 days after the inoculation (Fig. 5, A and B). The transfection with a control vector did not change the course of body weight loss. However, the transfection with IL-10 gene completely prevented a body weight loss and until 15 days after the inoculation when the mice inoculated with parental clone 20 or a clone 20/vector-1 cells lost body weight by more than 20%. Moreover, the mice inoculated with IL-10 transfecants exhibited neither a reduction in food intake nor severe hypoglycemia observed in mice implanted with either parental or clone 20/vector-1 cells (Fig. 5, B and C). Furthermore, the survival of the mice inoculated with IL-10 transfecant cells was significantly prolonged as compared with those implanted with parental cells (Fig. 6). These results demonstrated that IL-10 gene transduction can prevent colon 26-induced cancer cachexia effectively.
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The Effects of IL-10 Gene Transduction on the Pro-inflammatory Cytokine Expression in Vivo. Since IL-10 can inhibit production of several pro-inflammatory cytokines (10, 12, 13) that are presumed to be involved in the establishment of cancer cachexia (19–23), we examined the expression of pro-inflammatory cytokines at tumor sites and spleens from mice bearing either parental clone 20, clone 20/vector-1, clone 20/IL-10-1, or clone 20/IL-10-2 (Fig. 7). Transforming growth factor β transcript was detected at tumor sites and spleens in mice bearing a parental clone 20 cell or clone 20/vector-1. In contrast, IL-1β, TNF-α, and IFNγ transcripts were marginally detected at tumor sites and spleens from mice inoculated with clone 20 or clone 20/vector-1. IL-6 transcript was detected at tumor sites but not spleens of mice implanted with clone 20 or clone 20/vector-1. The transduction of IL-10 gene reduced significantly the amount of IL-6 transcript at tumor sites, whereas the mRNA expression of other cytokines at tumor sites did not change. Moreover, serum

Fig. 3. Serum IL-10 levels in mice inoculated with parental clone 20, clone 20/vector-1, clone 20/IL-10-1, or clone 20/IL-10-2 at 15 days after tumor implantation. Means are shown; bars, SD. Each symbol represents the value obtained from one animal. Statistical significance was evaluated by using Mann-Whitney.

Fig. 4. Local tumor growth rates. One million tumor cells were inoculated into the footpad of BALB/c mice. Tumor size was measured as the height every other day after the inoculation. Each group consists of five mice, and the results shown are representative of three independent experiments.

Fig. 5. Body weights (A), the amount of food intake (B), and blood glucose levels (C) after inoculation with various clones. A, body weights were measured every other day on each mouse inoculated with parental clone 20 (○), clone 20/vector-1 (●), clone 20/IL-10-1 (△), or clone 20/IL-10-2 (△). Each group consists of five animals, and the means were calculated; bars, SD. The representative results from three independent experiments are shown here. Statistical differences between two groups at each time point were
IL-6 levels in the mice bearing clone 20/IL-10-1 or clone 20/IL-10-2 were significantly lower than those bearing clone 20 or clone 20/vector-1 (Fig. 8). Because IL-6 was mainly involved in the establishment of cachexia in this model (7, 24), these results would indicate that IL-10 gene transduction reduced the production of IL-6 and concomitantly prevented cachexia.

Occurrence of Cachexia in IL-6-deficient Mice. If host cells such as monocytes/macrophages were a major source of IL-6, transfectant-derived IL-10 might inhibit directly IL-6 production by monocytes/macrophages. To examine this possibility, we injected parental clone 20 cells into IL-6-deficient BALB/c mice, which lack completely the ability to produce IL-6 (15). Parental clone 20 cells exhibited a similar growth rate in IL-6-deficient mice as in wild-type mice (Fig. 9A). Moreover, IL-6-deficient mice exhibited progressive weight loss (Fig. 9B) with elevated serum IL-6 levels (Fig. 9C) comparable to wild-type mice, indicating that tumor cells but not host cells are a major source of IL-6 in this model. Because IL-10 gene transduction failed to abrogate in vitro IL-6-producing capacity (Fig. 2), it is likely that IL-10, produced by the transfectant cells, might inhibit the functions of host cells, such as infiltrated monocytes, to produce IL-6-inducing factors, thereby reducing IL-6 production by tumor cells.

**DISCUSSION**

A crucial role of IL-6 in colon 26-induced cachexia was suggested by the previous analysis on pro-inflammatory cytokine expression in mice bearing two adenocarcinoma colon 26-derived clones, one cachexigenic clone 20, and the other non-cachexigenic clone 5 (7). Moreover, the administration of a neutralizing anti-IL-6 monoclonal antibody significantly reduced the weight loss induced by clone 20 cells (7, 24). Hence, the inhibition of action and/or production of IL-6 might generally alleviate cachexia in this model. This assumption was supported by the observation that suramin, originally developed as an anti-trypanosomal and anti-filarial agent, prevented partially colon 26-induced cachexia by interfering with IL-6 receptor binding (25).

IL-6 gene-deficient mice developed cachexia with increased serum levels of IL-6. In contrast, IL-6 gene-deficient mice exhibited higher levels of IL-6 than IL-6-deficient mice. These results indicate that IL-6 is a major factor in the development of cachexia in this model. The administration of a neutralizing anti-IL-6 monoclonal antibody significantly reduced the weight loss induced by clone 20 cells (7, 24). Moreover, the administration of suramin, originally developed as an anti-trypanosomal and anti-filarial agent, prevented partially colon 26-induced cachexia by interfering with IL-6 receptor binding (25).

**Fig. 6.** The survival rate of mice. The survival rate of mice inoculated with either clone 20/vector-1 (△; n = 15) or clone 20/IL-10-1 cells (○; n = 15) are shown here.

**Fig. 7.** Northern blot analysis of cytokine gene expression in spleens and tumor sites of mice bearing a parental and transfectant clones. Total RNAs were extracted from spleens and tumors of mice inoculated with parental or transfected clone 20 cells as described in "Materials and Methods." Northern blot analysis was performed to examine the expression of various cytokine genes. The representative results from two independent experiments are shown here.
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IL-6 levels when inoculated with parental clone 20 cells, establishing that tumor cells but not host cells are a major source of IL-6. However, several lines of evidence imply that colon 26 cells produced IL-6 in vivo only in response to pro-inflammatory cytokines, particularly IL-1, secreted by infiltrated monocytes (24, 26). In support of this notion, IL-1β transcript was detected consistently at the tumor sites of parental clone 20-bearing mice (7). However, the gene transduction of a natural IL-1 antagonist, IL-1ra, failed to abrogate its cachexigenicity, although transfectant cells produced a large amount of IL-1ra (7). Because more than a 1000-fold larger amount of IL-1ra is necessary to counteract the actions of IL-1 completely, the produced IL-1ra might not be sufficient to block the activities of locally produced IL-1. Alternatively, an additional monocyte-derived cytokine(s) or prostaglandin E2 (27) might be involved in regulating IL-6 production in vivo, and their action and/or production was not inhibited by IL-1ra. Because IL-1β transcript was scarcely reduced at the tumor sites of IL-10 transfectant-bearing mice as compared with parental clone-bearing ones, the IL-10 gene might inhibit the production of these mediators, thereby reducing IL-6 production.

IL-10 in vitro stimulates proliferation and differentiation of CD8⁺ cytolytic cells (28). Moreover, the transduction of IL-10 gene into tumor cell lines reduced their tumorigenicity by inducing peritumor CD8⁺ lymphocyte infiltration (29) or enhancing antibody-dependent immune response (30). On the contrary, in cutaneous basal cell carcinomas and B-cell lymphomas, tumor-derived IL-10 was presumed to depress local immune responses mediated by T cells, thereby promoting tumor progression (31, 32). However, we did not observe any difference in in vivo tumor growth rates and lymphocyte infiltration between a parental clone and IL-10 transfectant clones, suggesting that IL-10 gene transduction had little effects on the tumorigenicity of colon 26 cells.

Fig. 9. Local tumor growth rates (A), changes in body weights (B), and serum IL-6 levels (C). One million parental clone 20 cells were inoculated into the footpad of either IL-6-deficient BALB/c mice or wild-type BALB/c mice. Tumor size as height and body weights were measured every other day after the inoculation. IL-6 levels were determined by ELISA on sera taken 15 days after the inoculation. Each group consists of five mice. Statistical significance was determined using two-way ANOVA, followed by a multiple comparison.

Fig. 10. Presumed roles of the interaction between tumor cells and host cells in the establishment of cancer cachexia.

[Diagram showing IL-1Ra, IL-1, unidentified IL-6 inducer(s), colon26, IL-6, unidentified cachectin, Infiltrating MD, and cachexia.]

Statistical significance was determined using two-way ANOVA, followed by a multiple comparison.

4 Unpublished data.
The continuous administration of IL-6 failed to induce body weight loss (7), similarly as IL-6 transgenic mice did not exhibit body weight loss (33). Moreover, we demonstrated here that IL-10 gene transduction completely prevented cachexia, albeit an incomplete reduction of the elevated serum IL-6 levels. These results suggest the involvement of an additional factor(s) in the development of cachexia in this model. Recently, Todorov et al. (34) isolated a cancer cachectic factor from a mouse adenocarcinoma MAC16. The factor was identified as a Mr 24,000 proteoglycan with a core protein of 20 amino acids. The factor was present also in urine of cachectic cancer patients but not in normal subjects, patients with weight loss due to trauma, or cancer patients without weight loss (34). These results suggest that the production of this factor is under stringent control. Hence, it is tempting to speculate that IL-10 gene transduction may modulate the expression of this proteoglycan cachectic factor as well as IL-6, thereby preventing cachexia.

Several pro-inflammatory cytokines have been presumed to be involved in cancer cachexia. In addition, we have here demonstrated that one of the anti-inflammatory cytokines, IL-10, prevented colon 26-induced cachexia, although the efficacy of IL-10 gene transfer against cachexia caused by other tumors remains an open question. However, based on these observations, we presumed that the interaction between tumor cells and host cells, such as infiltrating macrophages, may be essentially involved in the establishment of cachexia caused by colon 26 cells (Fig. 10). The inflammatory reactions of host cells to tumor cells may be controlled by the gene therapy with other anti-inflammatory cytokines as well as IL-10 and may eventually prevent the development of cachexia, leading to improvement in the quality of life and prolongation of the survival.

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