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ORIGINAL ARTICLE

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Gene transfer of a hybrid interleukin-1β gene to B16 mouse melanoma recruits leucocyte subsets and reduces tumour growth in vivo

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Abstract Interleukin(IL)-1 differs from most other cytokines in its lack of a signal sequence. This results in intracellular retention of the immature proform. The release of IL-1 has been shown to be restricted predominantly to activated monocytes and macrophages and to be associated with apoptosis of the producer cell. These features have limited the investigation of IL-1 in early immune responses. In order to study the biological effects of local IL-1β release during an antitumour immune response, we used B16 mouse melanoma cells transduced with mature human IL-1β cDNA constructs. To obtain a released form of human IL-1β (ssIL-1β), the signal sequence from the related IL-1 receptor antagonist was ligated to the cDNA that encoded the mature form of IL-1β. When cells of the poorly immunogenic B16 melanoma cell line were transduced with IL-1β by retroviral infection, high levels of the protein were detected intracellularly, whereas cells transduced with IL-1β containing the signal sequence secreted most of their protein. The in vitro growth of the melanoma cells was unaffected by the IL-1β or ssIL-1β gene transfer. In contrast, the in vivo subcutaneous tumour growth of the ssIL-1β-transduced B16 cells in syngeneic C57BL/6 mice was significantly reduced compared with the IL-1β- and the mock-transduced controls. Immunohistochemical analysis revealed the infiltration of macrophages to be strong in B16/ssIL-1β, moderate in B16/IL-1β and minimal in control tumours. Furthermore, a moderate infiltration of $CD4^+$ cells and of scattered dendritic cells was detected in B16/ssIL-1β tumours whereas very few or no $CD4^+$ cells and dendritic cells were seen in the B16/IL-1β or control tumours. Following in vivo growth, all the tumours upregulated ICAM-1 on their cell surfaces. However, the percentage of ICAM-1-expressing cells was two- to fourfold higher in B16/ssIL-1β tumours compared to the control. The data suggest that IL-1β acts in vivo, either directly or indirectly, as a chemotactic factor for monocytes, T helper cells and dendritic cells. This supports IL-1β having a regulatory effect on tumour growth when locally released in the tumour area.

Key words IL-1β release · Signal sequence · Tumour growth inhibition

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Introduction

The transduction of tumour cells with certain cytokine genes, leading to localised release in the tumour, has been shown to be a highly efficient way to induce increased cytokine-mediated antitumour activity. Transduction of a number of different cytokines such as interleukin(IL)-1α [2], IL-2 [37], IL-4 [17], IL-7 [19], tumor necrosis factor α (TNFα) [4], interferon γ (IFNγ) [34], granulocyte/macrophage(GM)-colony-stimulating factor (CSF) [13] and others [8], into tumour cells has been shown to inhibit tumour growth in a number of different in vivo models. The rejection of established wild-type tumours has also been obtained through immunisation by tumour cells that secrete IL-2, IL-4, IFNγ, and GM-CSF [13, 17, 34, 37]. Targeting the expression and release of cytokines to the tumour is favourable since it improves the specificity and reduces the toxicity of the therapy compared with the systemic administration of cytokines. It also has the advantage of representing a more physiological mode of action since the action of most cytokines is paracrine (or autocrine) rather than endocrine.

IL-1β is one of the most pleiotropic and potent cytokines. It is produced mainly by activated monocytes and macrophages involved in inflammatory and immune re-

sponses [12]. The expression of IL-1β is strictly controlled by transcriptional regulation, posttranslational processing, release of soluble neutralising IL-1 type-II decoy receptors and accumulation of intra- and extracellular IL-1 receptor antagonists [3, 9, 12]. IL-1β is synthesised as a 31- to 34-kDa inactive precursor molecule. It is subsequently processed into a 17-kDa bioactive protein [12]. The fact that, in contrast to most other cytokines, IL-1β lacks a typical signal sequence has raised the question of how it is released. However, Hogquist and co-workers have obtained results showing both that IL-1β release is correlated with cell injury and that the processing of the precursor molecule only occurs efficiently in cells that are undergoing apoptosis [20, 21]. Apoptosis of human peripheral monocytes has been shown to be related to the IL-1β-converting enzyme [7, 30], which specifically cleaves the IL-1β precursor, releasing the mature form of the protein extracellularly [7, 22, 36]. The finding that the IL-1β-converting enzyme is homologous to the product of the *C. elegans* cell-death gene *ced-3* [30, 38] provides further support for the relation assumed between IL-1β release and apoptosis of the producer cell.

IL-1β has important pro-inflammatory and immunological properties, such as activating endothelial cells, increasing the expression of adhesion molecules in various cell types, costimulating T cell activation by increasing IL-2 receptor expression and inducing IL-2 production [12]. IL-1β also induces gene expression of a number of other cytokines such as IL-1, IL-4, IL-6, IL-8, TNFα, and TNFβ as well as enhancing natural killer cell activity [12]. These features make it tempting to speculate that IL-1β, when locally released in the tumour area, has the capacity of inducing antitumour activity. In a previous study [16], we developed a biological model in which IL-1β expression can be targeted extracellularly without affecting the viability of the producer cell. In order to obtain the released form of IL-1β, several cell lines were transduced with the mature human IL-1β cDNA fused to a signal sequence. The signal sequence used was derived from the structurally related mouse IL-1 receptor antagonist. The fusion of the signal sequence to the IL-1β gene (ssIL-1β) encoding the mature 17-kDa protein, resulted in the release of large amounts of IL-1β, whereas the mature IL-1β without the signal sequence (IL-1β) accumulated intracellularly. This model provides opportunities for evaluating the biological effects of IL-1β release during an antitumour immune response.

In the present study, we used the poorly immunogenic B16 F10 mouse melanoma cell line, transduced with IL-1β or ssIL-1β, to investigate whether the addition of a signal sequence influences tumour growth in vivo. We found that subcutaneous (s.c.) inoculations of ssIL-1β-transduced B16 melanoma cells in syngeneic mice resulted in reduced tumour growth and in prolonged survival compared with mock-transfected as well as tumour cells transfected with IL-1β without the signal sequence. The data suggest that inhibition of the growth of the genetically engineered tumour cells depends on the secretion of IL-1β, which either directly or indirectly functions as a chemotactic factor for macrophages, $CD4^+$ Th-cells and dendritic cells.

Materials and methods

Generation of IL-1β-producing mouse melanoma cells

The construction of the human IL-1β expression vectors used is described in detail by Gjörloff-Wingren et al. [16]. The GP+E-86 packaging cell lines [29] producing the different viruses were generated using Transfectam (Promega, Madison, Wis.) in accordance with the manufacturer's instructions. The B16 F10 cell lines [14], stably expressing the mature form of human IL-1β or ssIL-1β, were generated by retroviral infection as described previously [29]. In brief, 1×10^5 B16 F10 cells were seeded in 25-cm^2 flasks in complete medium (RPMI-1640; Gibco BRL, Life Technologies Inc., Paisley, Scotland) supplemented with 2 mM glutamine and 10% fetal calf serum and allowed to adhere overnight. A 1-ml sample of filter-sterilised viral supernatant, containing pLXSN (mock), pLXSN/IL-1β or pLXSN/ssIL-1β, was added to the cells together with polybrene (Sigma Chemical Co., St. Louis, Mo.), in a final concentration of 4 μg/ml. After 2 h at 37 °C, 4 ml complete medium was added and, 48 h later, the cells were selected in medium containing 0.8 mg/ml G418 (Gibco BRL, Life Technologies Inc., Gaithersburg, Md.). Confluent cells were harvested and seeded at 0.5 cell/well in 96-well plates so as to obtain clones. After 3–4 weeks, when clones were generated, freeze/thaw extracts of 5×10^6 cells and supernatants from these cells were analysed by the enzyme-linked immunosorbent assay (ELISA) technique [26] for IL-1β content. The clones expressing the highest levels of IL-1β were chosen for further studies. The clones were analysed regularly by ELISA for IL-1β production.

IL-1β ELISA

The IL-1β content in supernatants and in freeze/thaw extracts was assessed using the ELISA technique as described earlier [26]. In brief, 96-well assay plates were coated overnight at 4 °C with mAb ILB1-H6 (100 μl/well, 15 μg/ml) diluted in phosphate-buffered saline (PBS). The wells were washed with PBS/0.1% bovine serum albumin (BSA; Boehringer Mannheim, Germany) and were blocked with 200 μl 5% non-fat dry milk/PBS for 2 h at room temperature. After washing, 50 μl/well sample or human recombinant IL-1β standard and 50 μl of biotinylated anti-IL-B1-H67 (1 μg/ml) in 1% non-fat dry milk/PBS were added in duplicate and were incubated for 2 h at room temperature. After washing, 100 μl/well of a 1/3000 dilution of streptavidin-peroxidase (Vector Laboratories, Burlingame, Calif.) in 1% BSA/PBS was added to the wells and plates were incubated for 1 h at room temperature. After washing, 100 μl/well H_2O_2 and *o*-phenylendiamine dihydrochloride (Sigma Chemical Company, St. Louis, Mich.) in citrate buffer (pH 4.9) was added and the plates were incubated for 15–20 min, in the dark. Absorbance was measured at 450 nm by a Multiscan MC reader (Labsystem, Helsinki, Finland) and the samples were analysed by the software DELTA SOFT II (BioMetallics, Inc., N.J., USA). Measurement of IL-1β content was performed in the linear part of the standard curve.

Influence of IL-1β on in vitro tumour growth of B16 melanoma cells

The in vitro growth of the B16/IL-1β, B16/ssIL-1β and B16 mock-transfected cells was analysed by seeding 1×10^4 cells in 25-cm^2 flasks in complete medium. Thereafter, the cells were trypsinized and viable cells were counted every day for 5 days.

Animal model and tumour growth in vivo

Female C57BL/6 mice were obtained from Charles River, Sulzfeld, Germany, and Bomholtgaard, Ry, Denmark. Mice were kept under standardised conditions and were used when they reached the age of 10–18 weeks. Tumour growth was analysed by s.c. inoculations of different doses of viable B16/pLXSN, B16/IL-1β or B16/ssIL-1β cells, in the right flank of syngeneic mice. The cells were resuspended in

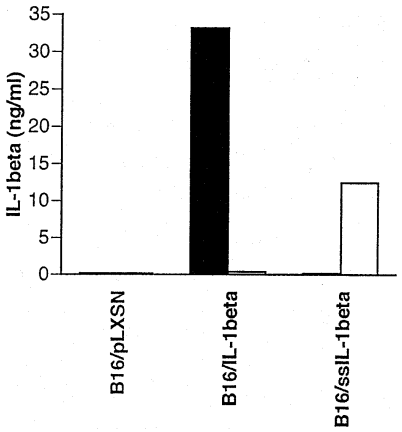

Fig. 1 The addition of a signal sequence to the interleukin(IL)-1β gene directs the protein extracellularly. Mock- and IL-1β-transduced B16 cells and B16 cells transduced with IL-1β containing the signal sequence (ssIL-1β) and supernatants thereof were harvested. Cell lysates (5×10^6 cells) were prepared by freezing and then thawing the cells. The cell lysates (■) and supernatants (□) were analysed for human IL-1β content by enzyme-linked immunosorbent assay. One representative of five experiments performed

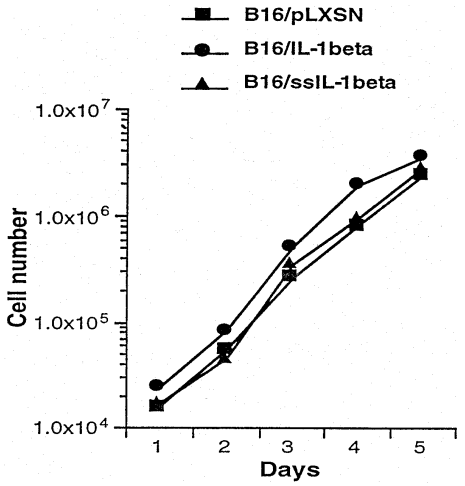

Fig. 2 In vitro growth of B16/pLXSN-, B16/IL-1β- and B16/ssIL-1β-transfected cells. Samples containing 1×10^4 cells were seeded in 25-cm^2 flasks, the cells being detached and counted on days 1, 2, 3, 4 and 5. One experiment of two similar experiments performed

0.2 ml PBS containing 1% syngeneic mouse serum. Tumour diameter was determined with a caliper two or three times/week. Animals were sacrificed when the tumour volume exceeded 1000 mm^3 or when the general condition of the animals was affected. The tumour volume was calculated by the formula: $V(mm^3) = 0.4 \times$ length (mm) $\times$ [width (mm)]^2.

Statistical analysis

The statistical significance of differences in tumour growth was assessed by the nonparametric Mann-Whitney *U*-test.

Immunohistochemical staining of tumour sections

Tumours and spleens from the sacrificed animals were removed and snap-frozen in isopentan (precooled to –55 °C). Cryostat sections of 5 μm were fixed in ice-cooled acetone and air-dried. Sections were blocked for 15 min in avidin, washed in PBS and then blocked for 15 min in biotin (Avidin/Biotin blocking kit; Vector Laboratories Inc.) before staining. The following primary antibodies were used: rat IgG2b anti-(mouse Mac-1) (clone M1/70) (Boehringer Mannheim, Mannheim, Germany); rat IgG2b anti-(mouse 1.B16.6) (previously described [24]); hamster IgG anti-(mouse N418) (kindly provided by Dr. R. Holmdahl, Department of Cell and Molecular Biology, Lund, Sweden); rat IgG2b anti-(mouse F4/80) (Serotec, United Kingdom); rat IgG2a anti-(mouse CD4) (clone RM4-5), rat IgG2a anti-(mouse CD8a) (clone 53-6.7), hamster IgG anti-(mouse CD54) (clone 3E2), rat IgG2b, κ anti-(mouse CD45), isotype control rat IgG2a anti-(mouse Ig), isotype control rat IgG2b anti-(mouse Ig) and isotype control hamster IgG anti-(mouse Ig), all from PharMingen, San Diego, Calif., USA. Staining with primary antibodies was followed by biotinylated goat anti-(rat IgG) or goat anti-(hamster IgG) (both from Jackson Immunoresearch Laboratories Inc., West Grove, Pa.) at a 1:400 or 1:300 dilution respectively, for 30 min. The sections were then incubated for 30 min with Vectastain ABC alkaline phosphatase (Vector Labs) or extrAvidin horseradish peroxidase (Sigma Immuno Chemicals). The antigen-antibody complexes were made visible by using either diaminobenzidine (Vector Labs) for 5 min or an alkaline phosphatase substrate kit I (Vector Labs) for 35 min. Finally, the slides were counterstained in hematoxylin and were mounted in DPX medium (KEBO Lab, Spinga, Sweden).

Collagenase treatment

Tumours from the sacrificed animals were treated with a collagenase medium in order to obtain single-cell suspensions. After removal of as much of the capsule as possible, the tumours were cut into small pieces and were transferred to 50 ml collagenase medium, containing collagenase (1 μg/ml), hyaluronidase (0.1 μg/ml) and DNase (0.02 μg/ml) diluted in complete medium without fetal calf serum. The mixture was rotated gently at room temperature for 1 h and then filtered through two layers of sterile cotton-weave compresses. The resulting cell suspension was further centrifuged on a fetal calf serum density gradient in order to remove cell debris. Finally, the cells were resuspended in complete medium and were either stained directly for flow-cytometric analysis or recultured in vitro for 2–4 weeks prior to flow-cytometric analysis.

FACS analysis of collagenase-treated tumours

Flow-cytometric analysis was performed using standard settings on a FACScan flow cytometer (Beckton Dickinson). The antibodies used: rat anti-(mouse 1.B16.6) mAb, hamster anti-(mouse CD54) conjugated with phycoerythrin, (PharMingen), and fluorescein-isothiocyanate-conjugated donkey anti-(rat IgG) F(ab′)$_2$, (Jackson Immunoresearch Laboratories Inc., West Grove, Pa.).

Results

Fusion of a signal sequence to the IL-1β cDNA directs the protein from cytoplasmic accumulation to extracellular release

The retroviral vector pLXSN containing the IL-1β or ssIL-1β gene constructs [16] was used to transduce B16 mouse melanoma cells by retroviral infection. The transduced cell lines were further cloned in order to obtain cells with a high production, as was described in Materials and methods. To evaluate whether the addition of a signal sequence influenced the secretion pattern, freeze/thaw extracts and super-

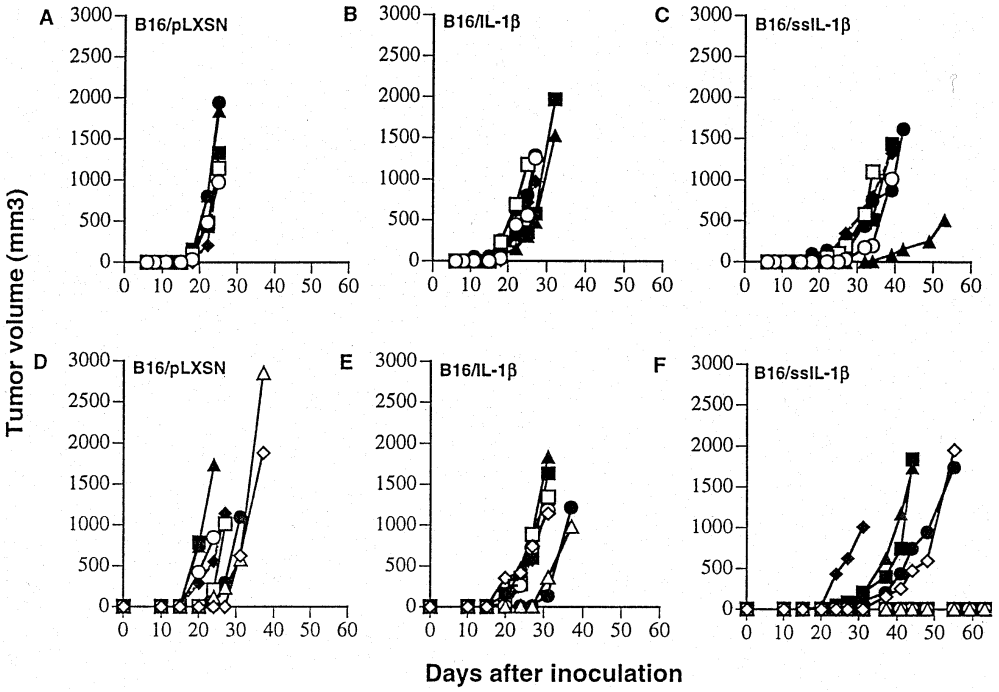

Fig. 3A–F In vivo growth of mock-, IL-1β- or ssIL-1β-transduced cells. Syngeneic C57BL/6 mice were inoculated s.c. in the right flank with 3×10^4 (**A–C**) or 1×10^4 (**D–F**) viable tumour cells. The growth of pLXSN-, IL-1β- and ssIL-1β-transduced tumour cells was followed over time, the tumour burden being measured two or three times weekly. Each line represents the individual tumour growth of one animal ($n = 6$ in **A–C** and $n = 8$ in **D–F**)

natants from the transduced cell lines were analysed for IL-1β content by the ELISA technique (Fig. 1). Cell lysates from the IL-1β-transduced B16 cells contained high levels of IL-1β, whereas very low levels were detected in the supernatant. In contrast, ssIL-1β-transduced B16 cells contained only marginal levels of IL-1β intracellularly but considerable amounts in the culture supernatants. No IL-1β was detectable in the mock-transfected control cells (B16/pLXSN).

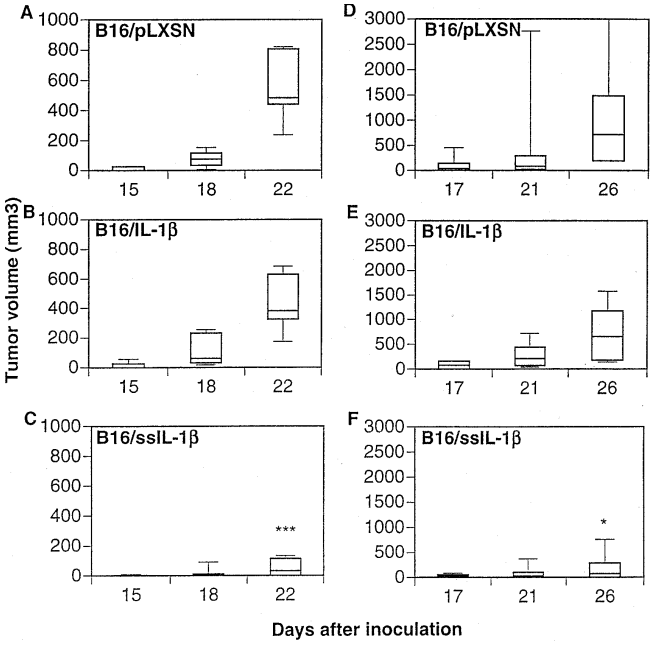

Fig. 4A–F IL-1β-secreting tumours show significant reduction in tumour growth. The tumour volumes are presented by box plots in which the boxes represent 50% of the measured values, the lines extending vertically from the boxes constituting the upper and the lower 25% of the measured values. Median tumour volumes after 15–22 (**A–C**) and 17–26 (**D–F**) days of tumour growth are shown. The animals were inoculated with 3×10^4 (**A–C**) or with 1×10^4 (**D–F**) cells. One representative experiment of three experiments performed. $^*P < 0.05$ versus both B16/pLXSN and B16/IL-1β; $^{***}P < 0.001$ versus both B16/pLXSN and B16/IL-1β

The expression of IL-1β does not influence the in vitro cell growth

In order to determine whether gene transfer of the IL-1β constructs affects cell growth in vitro, 1×10^4 cells were seeded in culture flasks and detached and the cells were counted after 1, 2, 3, 4 and 5 days. As can be seen in Fig. 2, the mock-, IL-1β- and ssIL-1β-transduced cells showed similar in vitro growth. The in vitro cell growth was also analysed by measuring the DNA synthesis as [^{3}H]thymidine uptake. The results confirmed that the expression of IL-1β, either intracellularly or extracellularly, did not influence the in vitro cell proliferation (data not shown).

IL-1β release in the tumour area mediates reduced tumour growth in vivo

To examine the relation between IL-1β release in the tumour area and tumour growth in vivo, syngeneic C57BL/6 mice were inoculated s.c. with either B16/pLXSN, B16/IL-1β or B16/ssIL-1β cells. Tumour cells inoculated into mice at 3×10^4 (high) cells/animal or 1×10^4 (low) cells/animal resulted in distinct growth characteristics of the transfectants. There was a marked reduction in tumour growth rate in the mice receiving B16/ssIL-1β transfectants as compared with mice inoculated with mock- or IL-1β-transduced tumour cells, although the

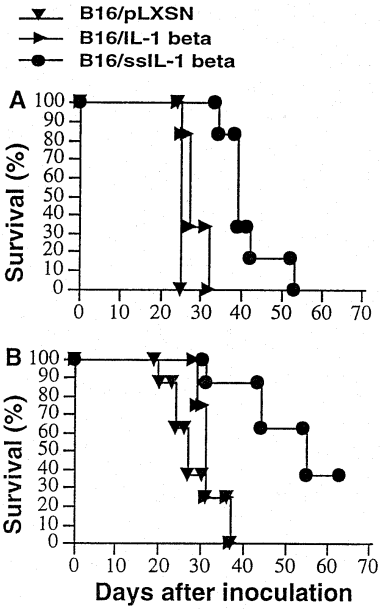

Fig. 5A,B The reduction in tumour growth of IL-1β-secreting tumours correlated with the prolongation of survival. The animals were sacrificed when the tumour burden exceeded 1×10^3 mm^3. The tumours were induced by s.c. injections of 3×10^4 (**A**) or 1×10^4 (**B**) B16/pLXSN, B16/IL-1β or B16/ssIL-1β cells in the flank of syngeneic mice (six to eight mice/group). One representative experiment of three experiments performed

difference was less pronounced when the higher tumour cell dose was administered [Fig. 3A–C (high) and D–F (low)]. The low dose resulted in tumours reaching a volume of approximately 500 mm^3 between days 20 and 30 in the control group and days 25 and 30 in the B16/IL-1β group.

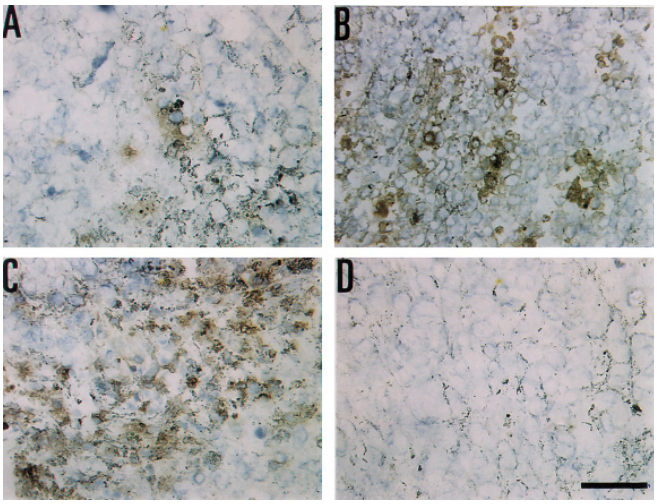

Fig. 6A–D Strong infiltration of macrophages in the tumour areas of IL-1β-secreting tumour cells. **A** B16/pLXSN, **B** B16/IL-1β and **C** B16/ssIL-1β were stained with the mAb MAC-1 (5 μg/ml). **D** The staining of B16/ssIL-1β with an isotype-matched (IgG2b) control antibody (5 μg/ml). The bound antibodies were made visible by use of diaminobenzidine. Sections were counterstained in hematoxylin. The positively stained cells are brown. (*Scale bar* 50 μm, valid for **A–D**)

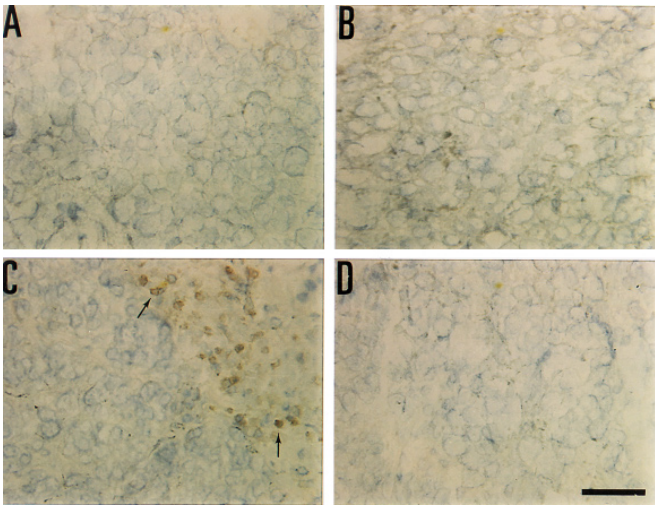

Fig. 7A–D Infiltration of dendritic cells in tumours secreting IL-1β. The sections were stained with the dendritic-cell-related mAb N418 (5 μl/ml). **A** B16/pLXSN, **B** B16/IL-1β, **C** B16/ssIL-1β, **D** the staining of B16/ssIL-1β with a hamster IgG isotype-matched control antibody (5 μg/ml). The bound antibodies were made visible by use of diaminobenzidine. Sections were counterstained in hematoxylin. The positively stained cells (*arrows*) are brown. (*Scale bar* 50 μm, valid for **A–D**)

In contrast, in the mice inoculated with IL-1β-secreting B16 transfectants, the same tumour volume was reached approximately between days 40 and 45 (Fig. 3D–F). The tumour volume in animals receiving 1×10^4 cells was almost tenfold reduced on day 26 in the B16/ssIL-1β group compared with the B16/pLXSN and B16/IL-1β groups ($P < 0.05$, Fig. 4D–F). Similarly, on day 22 the tumour

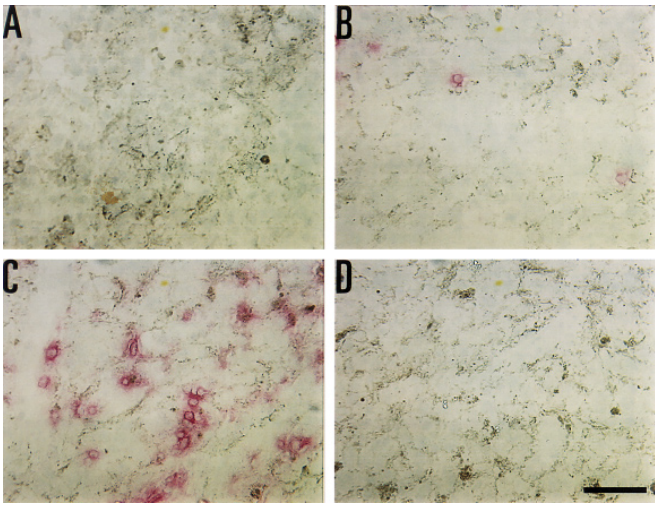

Fig. 8A–D IL-1β-secreting tumour cells induce a moderate infiltration of CD4$^+$ cells in focal areas of the tumour. **A** B16/pLXSN, **B** B16/IL-1β, and **C** B16/ssIL-1β were stained with the anti-CD4 mAb (5 μg/ml), **D** shows the staining of a section from a B16/ssIL-1β tumour with anti CD8 mAb. The anti-CD8 stainings were similar for all the tumour transfectants (data not shown). The bound antibodies were made visible by the use of Vector′s red substrate. Sections were counterstained in hematoxylin. The positively stained cells appear as red. (*Scale bar* 50 μm, valid for **A–D**)

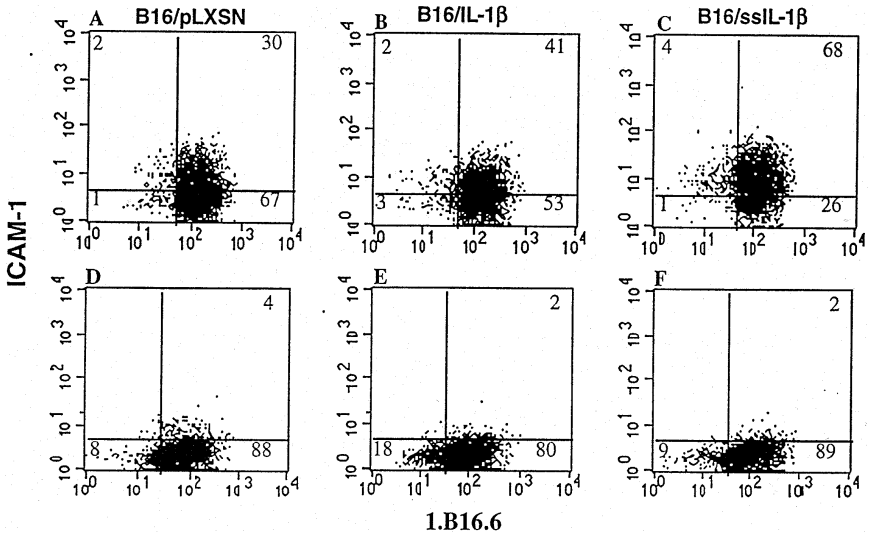

Fig. 9A–D The frequency of ICAM-1-expressing tumour cells is elevated in the in vivo grown B16/ssIL-1β cells as compared with the in vivo grown B16/pLXSN cells. Single-cell suspensions of the tumours were prepared and were analysed by FACS. Tumours were removed from the animals when the tumour volume exceeded 1×10^3 mm^3. **A–C** The tumour cells being analysed for ICAM-1 expression after growth in vivo and thereafter being recultured for 3 weeks in vitro. **D–F** In vitro cultured tumour cells; one representative experiment of three similar experiments performed

volume was significantly reduced in animals inoculated with 3×10^4 B16/ssIL-1β cells compared with those inoculated with mock- or IL-1β-transduced B16 cells ($P < 0.001$, Fig. 4A–C).

The reduced in vivo tumour growth rate of the B16/ssIL-1β tumours correlated with prolonged survival in animals receiving 3×10^4 (high) or 1×10^4 (low) cells (Fig. 5A,B). Approximately 30% of the animals challenged with 1×10^4 cells remained tumour-free after 60 days whereas none of the animals remained tumour-free after inoculation of B16/pLXSN or B16/IL-1β cells (Fig. 5B).

Thus, genetic manipulation of the parental B16 melanoma cells to secrete IL-1β seems to convert this aggressive and poorly immunogenic tumour into a less tumorigenic variant.

Phenotype of tumour-infiltrating cells in response to local IL-1β secretion

In order to investigate whether the reduced tumorigenicity correlated with an increase in the infiltration of leucocytes, immunohistochemical analysis of tumour biopsies was performed. In the mock-transfected tumours, very limited numbers of $CD45^+$ leucocytes were detected whereas in the B16/IL-1β and B16/ssIL-1β tumours a substantial infiltration was recorded (data not shown). Staining the tumour sections with the mAb 1.B16.6, which specifically reacts with B16 melanoma cells [24], confirmed that the biopsies contained melanoma cells (data not shown). A strong infiltration of $MAC\text{-}1^+$ macrophages was recorded in the B16/ssIL-1β tumours (Fig. 6C), whereas a moderate infiltration was observed in the B16/IL-1β tumours (Fig. 6B). Moreover, in the B16/ssIL-1β tumours, $MAC\text{-}1^+$ cells infiltrated the entire tumour, whereas only local areas stained positively for MAC-1 in the B16/IL-1β tumours. The mock-transfected tumours contained only marginal numbers of $MAC\text{-}1^+$ cells (Fig. 6A). To verify that the cells staining positively for MAC-1 were macrophages, the mAb F4/80 was used. The staining pattern obtained with F4/80 correlated with the MAC-1 staining, confirming the recruitment of macrophages to the B16/IL-1β and B16/ssIL-1β tumours (data not shown). Furthermore, to determine whether dendritic cells were present in the tumour area, tumour sections were stained with the dendrite-related mAb N418. The staining with N418 mAb was significant in ssIL-1β-transduced tumours but was absent in mock- and IL-1β-transduced tumours (Fig. 7A–C). Moreover, a moderate infiltration of $CD4^+$ T helper cells was detected in the ssIL-1β-secreting tumours whereas very few $CD4^+$ T cells were present in the IL-1β-or mock-transduced tumours (Fig. 8A–C). No $CD8^+$ cells were detected in any of the tumours (Fig. 8D). In contrast, a strong $CD8^+$ staining was observed in the spleens of all the animals investigated (data not shown).

Up-regulation of ICAM-1 on tumour cells in vivo

In order to analyse the expression of surface antigens on the various tumour cells after in vivo growth, we prepared single-cell suspensions from tumour biopsies and performed flow-cytometric analysis. An up-regulation of ICAM-1 was detected on the cell surface of all the three B16-transduced tumours after in vivo growth. This up-regulation was constitutive and remained elevated even after 3 weeks of in vitro reculture (Fig. 9A–C), as compared with cells cultured in vitro (Fig. 9D–F). Interestingly, the percentage of ICAM-1-expressing $1.B16.6^+$ tumour cells in the IL-1β-secreting B16 transfectants was elevated more than twofold compared with the mock transfectants. The percentage of B16/IL-1β tumour cells expressing ICAM-1 was elevated only slightly. Analysis of the expression of MHC class I and II, which was marginal or absent in the in vitro cultured B16 cells, failed to show any modulation after in vivo growth (data not shown). Moreover, expression of neither B7.1 nor B7.2 was recorded on the tumour cells after in vivo growth. In contrast, almost all the tumour-infiltrating $MAC\text{-}1^+$ macrophages expressed both B7.1 and B7.2 (data not shown).

Discussion

IL-1β is one of the most pleiotropic cytokines possessing both potent proinflammatory and immunostimulatory properties [12]. IL-1 is a cytokine that can elicit such multiple antitumour mechanisms as the enhancement of cytotoxic activity of monocytes [31], natural killer cells and T lymphocytes [11]. Moreover, IL-1 mediates increased expression of several cytokine genes, including IL-2, IL-4, and GM-CSF [12], that have been shown to have antitumour activity [13, 17, 37]. It has also been proposed that IL-1β exerts direct cytotoxic effects on various tumour cells [28, 31]. However, in most of the studies performed, the cytokine has been applied systemically, which often is accompanied by detrimental effects on the host, such as fever, anorexia, neutrophilia and hypotension [35].

We have demonstrated here that the release of a genetically modified IL-1β locally in the tumour area has the potential of converting an aggressive, poorly immunogenic tumour into a less tumorigenic form. IL-1 lacks a signal peptide [5] and the mechanism for the release of IL-1 is still elusive. However, recent results obtained by Hogquist and co-workers [20, 21] suggest that the release of it is associated with cell injury and that the processing of the precursor molecule only occurs efficiently in cells that are undergoing apoptosis. In a previous study [16], we showed that the transfection of NIH 3T3 cells with the mature form of human IL-1β cDNA that was fused to a signal sequence, directed the protein from cytoplasmic accumulation to the secretory pathway. This resulted in an excessive release of IL-1 without the viability of the producer cell being affected. Thus, genetically modified IL-1 allows the producer cell to act as an accessory cell with the ability to regulate a long-lasting immune response. In the present study, we extend the scope of the previous study through the inclusion of IL-1β-transduced melanoma cells, which provides a model for in vivo tumour-biological studies. The primary reason for using human IL-1β was to be able to distinguish the introduced gene product from endogenous murine IL-1β. There is an extensive sequence homology between the mouse and human IL-1β gene product and human IL-1β shows strong bioactivity against both mouse and human T cells. The different B16 clones used in this study all expressed low amounts of MHC class I and undetectable amounts of MHC class II. Moreover, transfection of B16 melanoma with an irrelevant human gene construct did not induce a change in the growth pattern (data not shown). Thus, it seems highly likely that the results in this study are entirely reflecting the release of bioactive IL-1 in the tumour area and that potential immune reactions against the human product will not have influenced these results to any major extent.

The transfection of ssIL-1β to B16 melanoma cells resulted in a significant inhibition of tumour growth (Figs. 3, 4), which correlated with prolonged survival. Indeed, about 30%–40% of the animals inoculated with 1×10^4 B16/ssIL-1β cells remained tumour-free after more than 60 days. In contrast, control tumours grew progressively, all of these animals being moribund after 30 days. The reduction in tumour growth rate in the mice receiving B16/ssIL-1β transfectants was not due to clonal effects, since different B16/ssIL-1β clones showed similar reduced growth compared to control B16-transfected cells (data not shown). The ssIL-1β-transduced tumours seemed not to mediate any IL-1β-related side-effects, the levels of IL-1β in the serum from these animals being undetectable (data not shown). This can be explained by IL-1β activity being strictly controlled at different levels, including the protein and the receptor level [9]. Colotta and co-workers have shown that several chemotactic agents, including C5a, platelet-activating factor, and IL-8, cause a rapid reduction in the IL-1-binding capacity of human polymorphonuclear leucocytes through the release of IL-1 type-II receptors [10]. Thus, chemoattractant-induced IL-1 type-II decoy receptors, released systemically, may limit the endocrine effects of IL-1 while preserving the capacity of the immunocompetent cells to respond to paracrine IL-1 secretion at the tumour site. It is, therefore, tempting to speculate that most of the IL-1β released into the circulation is neutralised by soluble IL-1 type-II decoy receptors. Alternatively, the tumour cells themselves may produce some as yet unidentified IL-1β-neutralising factor.

Growth inhibition seemed to correlate with the ability of the secreted IL-1β to induce the massive infiltration of monocytes, together with the moderate recruitment of $CD4^+$ T helper cells and dendritic cells into the tumour area. The finding that the modulation of tumour growth by ssIL-1β in immunodeficient nude mice was less than the reduction in tumour growth observed in immunocompetent syngenic mice (data not shown) indicates that the $CD4^+$ T helper cells may play a crucial role in the inhibition of growth. The dendritic cells detected in the ssIL-1β-transfected tumours may also play a prominent role in the inhibition of tumour growth, since these are the most potent antigen-presenting cells, expressing high levels of MHC I and MHC II, as well as the costimulatory molecules B7.1 and B7.2. It has previously been shown that dendritic cells may be successfully used in several tumour vaccination protocols [23, 27]. No significant growth inhibition was observed when tumour cells were transduced with IL-1β without a signal sequence, despite the substantial infiltration of monocytes in the tumour. It can be speculated that minimal tumour cell death occurs during the initial growth of a tumour, resulting in marginal levels of released IL-1β. As the tumour grows, leakage of IL-1β from necrotic tumour cells occurs and induces infiltration of blood monocytes. However, by this time the tumour can have progressed to a stage in which it is unaffected by the immune response. Early IL-1β secretion may be crucial to mediating an early inflammatory response that results in the recruitment of $CD4^+$ T cells and dendritic cells. It is well known that IL-1 induces the expression of several adhesion molecules, such as ICAM-1 [33], ELAM-1 [6] and VCAM-1 [32], on various target cells. Furthermore, IL-1 is a stimulator of cytokine expression in both T cells and macrophages. Several of the cytokines induced by IL-1, such as TNFα, IL-2, IL-4, and GM-CSF [4, 13, 17, 37], are known to have antitumour

activity. The pattern of endogenous cytokines produced in the tumour area is currently being investigated immunohistochemically.

FACS analysis of disaggregated cells from tumours revealed a strong up-regulation of ICAM-1 on ssIL-1β-transduced tumour cells grown in vivo. The up-regulation was constitutive and remained even after 3 weeks of in vitro reculturing. ICAM-1 has been shown to have costimulatory effects on T cells and to be essential when the levels of the MHC II/Ag peptide complex are low [1, 15]. However, the augmented expression of ICAM-1 on tumour cells has also been found to be associated with an increased risk of metastasis [18, 25]. Thus, ICAM-1 seems able to exert dual effects in tumour development and progression.

Apte and co-workers have demonstrated reduced tumorigenicity of IL-1α-producing cell lines [2, 39]. They also detected an immunological memory in conjunction with the rejection of IL-α-producing fibrosarcomas. In contrast, Gilboa and co-workers reported the gene transfer of the proform of IL-1α and IL-1β to have only weak effects in the therapeutic vaccination of bladder cancer. However, since the proform of IL-1β has to be processed in order for it to express biological activity, and since not all cell types express IL-1β-converting enzyme, it is likely that these cells were unable to cleave the proform. Alternatively, since our transfectants only marginally released IL-1β in the absence of a signal sequence, the cells may have failed to secrete the protein.

Our results suggest that IL-1β possesses antitumour activity and acts, either directly or indirectly, as a chemotactic factor for macrophages, $CD4^+$ T cells and dendritic cells in vivo. This supports IL-1β having a regulatory role during a local antitumour response. The targeted extracellular delivery of mature IL-1β protein provides a unique approach to evaluating the importance of IL-1β expression for a primary antitumour immune response. Currently we are investigating the effects of combining IL-1 and IL-2 gene transfer in efforts to enhance the IL-1β-induced antitumour response. Such an approach may serve to recruit and expand the $CD4^+$ and $CD8^+$ T cells further at the tumour site.

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