GROWTH AUTONOMY AND TUMORIGENICITY OF INTERLEUKIN 6-DEPENDENT B CELLS TRANSFECTED WITH INTERLEUKIN 6 cDNA

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IL-6 is a pleiotropic cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes, and T cells. While this molecule has been identified independently in several different bioassays as B cell stimulatory factor 2, IFN-β2, 26-kd protein, plasmacytoma growth factor, and hepatocyte stimulating factor, the amino acid sequence of proteins, the molecular cloning of the corresponding genes, and the availability of recombinant proteins have led to the conclusion that all the biological activities can be attributed to one and the same polypeptide, now called IL-6 (reviewed in references 1-3). This glycoprotein induces the final maturation of B cells into Ig-secreting cells (4), the proliferation of plasmacytomas, B cell hybridomas, and EBV-transformed B lymphoblastoid cells (5-9), the IL-2 production, proliferation, and differentiation of T cells (10-13), the proliferation and differentiation of hematopoietic precursors (14), and the production of acute phase proteins by hepatocytes (15).

It is well known that plasmacytomas can be induced in a high percentage of BALB/c mice after the intraperitoneal injection of mineral oil or pristane, which evokes the formation of a chronic granulomatous tissue that consists primarily of macrophages and neutrophils (16). A macrophage-derived factor has been demonstrated to be essential for in vitro survival and proliferation of murine plasmacytomas, suggesting the involvement of the factor in the establishment and maintenance in vivo of pristane-induced murine plasmacytomas (17-19). The determination of amino acid sequence of the plasmacytoma growth factor and the cloning of a corresponding gene revealed that the factor was a murine equivalent of human IL-6 (5, 8, 20). In humans, the involvement of IL-6 has been also suggested in the malignant transformation of B cells. Myeloma cells freshly isolated from patients with multiple myeloma produced and responded to IL-6, and anti-IL-6 antibody inhibited their in vitro proliferation. These observations led to the proposition that an IL-6 autocrine mechanism might operate in oncogenesis of human myelomas (21).

The generation of cells that constitutively express IL-6-R and secrete IL-6 should provide a model system to assess the role of IL-6 as an autocrine growth factor in
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B cell malignancy. In the following study, we present the results obtained after introduction of an expression vector carrying an IL-6 cDNA into an IL-6-dependent murine B cell line. The transfectants proliferated in vitro autonomously through an IL-6 autocrine mechanism and were found to be highly tumorigenic in vivo. Our results indicate that the autocrine action of IL-6 is of importance in the process of multistage oncogenesis of B lineage cells.

Materials and Methods

Plasmid Construction. A 650-bp-long BglII-BanI fragment of the plasmid pSP64-T 26K carrying a human IL-6 cDNA (22) was rendered blunt-ended, ligated to XhoI linkers, and cloned into the XhoI site of the expression vector BCMGNeo (23) in the proper orientation. In the resulting plasmid, BCMGNeo IL-6, the transcription of IL-6 cDNA, from which most of the 3' untranslated region containing A and T nucleotide-rich sequences responsible for the destabilization of transcripts has been deleted, is under the control of the promoter/enhancer of human CMV.

Preparation of Human rIL-6. Cell lines secreting a large quantity of human rIL-6 were established by transfection of Ig-nonproducing murine myeloma X63-Ag8.653 cells (24) with the IL-6 cDNA expression vector BCMGNeo IL-6, as described previously (25). The media conditioned by G418-resistant stable transfectants contained >10^5 U/ml of IL-6 activity and were used as the source of rIL-6.

Preparation of Polyclonal Anti-human IL-6 Antibodies. Human rIL-6, prepared from culture supernatants of the IL-6 cDNA-transfected X63-Ag8.653 cells by SDS-PAGE, was mixed with CFA and injected subcutaneously into rabbits at 2-wk intervals for 2 mo to obtain an antiserum against human IL-6. Serum from preimmunized rabbits was used as control.

Establishment of IL-6-dependent Cell Lines. BALB/c mice (Japan SLC Inc., Hamamatsu, Japan) were injected intraperitoneally with 50 μg of Escherichia coli 055:B5 LPS (Difco Laboratories, Detroit, MI) 3 d before fusion of their spleen cells with X63-Ag8.653 myeloma cells. Among hybridomas growing in media containing HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) and 500 U/ml of rIL-6, those of which survival and proliferation were absolutely dependent on exogenously supplied IL-6 were selected and cloned by the limiting dilution method. These IL-6-dependent cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY), 100 U/ml of penicillin-streptomycin (Gibco Laboratories), 2 mM L-glutamine (Gibco Laboratories), 5 x 10^-5 M 2-ME, and 500 U/ml of rIL-6.

DNA Transfection. DNA transfection was performed by the protoplast fusion technique (26). Bacteria bearing the plasmid BCMGNeo IL-6 were converted to protoplasts and fused to an IL-6-dependent cell line (F12-28) by using polyethylene glycol 2,000 (Wako Pure Chemical Industries, Osaka, Japan). 2 d after the fusion, cells were cultured in the presence of 0.8 mg/ml of G418 (Gibco Laboratories) and 500 U/ml of rIL-6. Among G418-resistant transfectants, those grown even in the absence of exogenous IL-6 were selected and then subcloned by the limiting dilution method. Subclones of transfectants were thereafter maintained in media without G418 and exogenous IL-6.

Assay for IL-6 Activity. IL-6 activity was measured by the [3H]thymidine incorporation of an IL-6-dependent cell line (B45-3) established by fusion of rat spleen cells with murine B cell hybridoma SP2/0 cells (N. Tohyma et al., unpublished observations). Serial dilutions of IL-6 were incubated with 5 x 10^3 B45-3 cells in a volume of 0.2 ml for 48 h, followed by a 6-h pulse of 1 μCi [3H]thymidine (Amersham Japan, Tokyo, Japan). 1 U of activity was defined as the amount of IL-6 that induced 50% of maximal proliferation in a 0.2-ml culture.

Cell Proliferation Assay. Cells were cultured at 2 x 10^4 cells/0.2 ml (Figs. 1 and 3) or at indicated densities shown in Fig. 4, in the presence or absence of rIL-6 for 72 h (Figs. 1 and 3) or for 48 h (Fig. 4), followed by a 6-h pulse of 1 μCi of [3H]thymidine, and [3H]thymidine incorporation was measured by liquid scintillation counting. In the growth inhibition assay, rabbit anti-IL-6 antiserum or preimmune serum was added at the start of the culture.
Cell growth was also measured by counting cell numbers. Cells were seeded at $5 \times 10^3$ in a 0.2-ml culture in the presence or absence of rIL-6 (500 U/ml). Number of viable cells was determined every 12 h up to 72 h by the trypan blue dye exclusion method.

**Tumorigenicity Assay.** F12-28 cells untransfected and transfected with BCMGNeo or BCMGNeo IL-6 were washed with and resuspended in PBS at a concentration of $10^6$ cells/ml. Groups of four or five BALB/c mice (6-7 wk old; Japan SLC Inc., Hamamatsu, Japan) and BALB/c nude mice (6-7 wk old; CLEA Japan Inc., Tokyo, Japan) were injected subcutaneously into the axillae with $10^6$ cells. Mice were monitored every 5 d for the appearance of solid tumors.

**Results**

**Establishment of an IL-6-dependent Cell Line (F12-28).** Spleen cells from BALB/c mice injected with LPS were fused with Ig-nonproducing myeloma X63-Ag8.653 cells derived from BALB/c mice, and HAT selection was performed in the presence of exogenously supplied rIL-6. While the majority of B cell hybridomas established under this condition were able to grow even in the absence of exogenous IL-6 (Fig. 1 B), very few clones turned out to be absolutely dependent on exogenous IL-6 for their survival and proliferation. F12-28, one of the subclones derived from these IL-6-dependent clones, was further characterized and used for the following studies. F12-28 cells responded by proliferation to rIL-6 in a dose-dependent manner (Fig. 1 A) and died within 72 h, unless rIL-6 was supplied (Fig. 2 A).

**Autonomous Growth of IL-6 cDNA-transfected F12-28 Cells.** An IL-6 cDNA expres-
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Expression vector (BCMGNeo IL-6) was constructed by inserting a cDNA encoding human IL-6 (22, 27) into the bovine papilloma virus-based vector BCMGNeo (23). To enhance the expression, most of the 3' untranslated region containing A and T nucleotide-rich sequences responsible for the destabilization of transcripts (28, 29) has been deleted from the cDNA. In BCMGNeo IL-6, the promoter/enhancer of human CMV, and the splicing/poly(A) addition signals of the rabbit β globin gene were placed for the transcription of IL-6 cDNA; in addition, the neomycin resistance gene was inserted for the selection of stable transfectants. Though human IL-6 cDNA is 35% different from murine IL-6 cDNA at the nucleotide sequence and 58% at the deduced amino acids (20), human IL-6 is active on mouse cells (7).

To generate cell lines of B lineage that constitutively express IL-6-R and secrete IL-6, an IL-6-dependent cell line (F12-28) was transfected with BCMGNeo IL-6 by means of the protoplast fusion technique (26). Among G418-resistant stable transfectants, ~80% of clones were able to grow in the absence of exogenously supplied IL-6 (Fig. 2 C), while any of F12-28 cells transfected with the vector BCMGNeo alone were not (Fig. 2 B). The IL-6 cDNA transfectants grown without any requirement for exogenous IL-6 were subcloned, and five independent clones, IL-6A2, IL-6A6, IL-6A14, IL-6B2, and IL-6B8, were established for further analysis (Table I).

To elucidate whether the autonomous growth of IL-6 cDNA-transfected F12-28 cells is mediated by an IL-6 autocrine mechanism, we first examined the presence of IL-6 activity in the culture supernatant of the transfectants. The IL-6 cDNA-transfected F12-28 cells synthesized and secreted IL-6 ranging from 70 to 880,000 U/10⁶ cells/ml in a 48-h culture, whereas no detectable IL-6 was found in media conditioned by F12-28 cells untransfected or transfected with the vector alone, or the fusion partner X63-Ag8.653 or in the media of an IL-6-independent B cell hybridoma (C2) (Table I). Northern blot analysis revealed that the IL-6 cDNA transfectants expressed human IL-6-specific transcripts carrying the sequence derived from the expression vector, indicating that the IL-6 production of the transfectants was caused by the introduced human IL-6 cDNA and not by the activation of an endogenous IL-6 gene (data not shown).

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<th>Secretion of IL-6 by IL-6 cDNA-transfected F12-28 Cells</th>
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<tr>
<td>X63-Ag8.653</td>
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<tr>
<td>C2 (B hybridoma)</td>
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<td>F12-28</td>
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<tr>
<td>Vec3</td>
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Media conditioned for 48 h by cells (10⁶/ml) were tested for IL-6 activity on an IL-6-dependent cell line B45-3 by [³H]thymidine incorporation (see Materials and Methods). 1 U of activity was defined as the amount of IL-6 that induced 50% of maximal proliferation of B45-3 cells.
Anti-IL-6 Antibodies Inhibit the Proliferation of IL-6 cDNA Transfectants. We next examined whether secreted IL-6 played a role as an autocrine growth factor for the proliferation of IL-6 cDNA transfectants. For this purpose, polyclonal antibodies to IL-6 were prepared by injecting rabbits with purified human rIL-6. The serum from immunized rabbits completely inhibited the proliferation of IL-6-dependent F12-28 cells stimulated with rIL-6 (Fig. 3A). The inhibition of proliferation was totally abolished by the addition of excess rIL-6. In contrast, no growth inhibition was caused by the serum from preimmunized rabbits. Moreover, the serum from immunized rabbits had no effect on the proliferation of the IL-6-independent B cell hybridoma C2 (Fig. 3B). These results confirmed that the inhibitory effect of the antiserum was due to the specific activity against IL-6. The proliferation of IL-6 cDNA transfectants was suppressed by the addition of the anti-IL-6 antiserum; the growth inhibition was almost complete in case of lower producers of IL-6 such as IL-6A14 (Fig. 3C) and IL-6B8, partial in IL-6A6, and little or none in higher producers such as IL-6A2 and IL-6B2 (data not shown). This observation indicated that IL-6 secreted by IL-6 cDNA transfectants was essential for their autonomous growth.

Proliferation of IL-6 cDNA Transfectants Is a Function of Cell Density. The transfectant IL-6A14 cells maintained at relatively high density (2–5 × 10^5 cells/ml) were replated at various cell densities ranging from 10^3 to 1.6 × 10^4 cells/ml in the presence or absence of exogenous IL-6, and their proliferation was determined by [3H]thymidine incorporation. The proliferation rate, expressed as [3H]thymidine incorporation per cell, declined as the cell density became lower in the absence of exogenous IL-6 (Fig. 4B). However, the addition of rIL-6 rendered the rate constant regardless of cell density, as observed in untransfected or mock-transfected F12-28 cells stimulated with rIL-6 (Fig. 4A). This result suggested that the autonomous

![Figure 3. Anti-IL-6 antibodies inhibit the proliferation of IL-6 cDNA-transfected F12-28 cells.](image-url)
growth of the transfectants required accumulation of autogenous IL-6 in the surrounding media. Indeed, the dependency of proliferation on cell density was more prominent in lower producers of IL-6 than in higher producers. The growth of the lowest IL-6 producer IL-6B8 cells was greatly slowed, and they finally died when they were maintained at too low a density (data not shown).

Enhanced Tumorigenicity of IL-6 cDNA Transfectants. All the growth properties of the IL-6 cDNA transfectants shown above indicated that the production of IL-6 enabled IL-6-dependent cells to acquire growth autonomy in vitro through an autocrine self-stimulation. To assess the tumorigenicity of these cells in vivo, BALB/c nude mice were injected subcutaneously with 10^5 cells of each clone and monitored for tumor appearance and growth. As shown in Table II, the IL-6 cDNA transfectants expressing high levels of IL-6, such as IL-6A2, IL-6B2, and IL6-A6, formed growing tumors as early as 5 d after inoculation. All animals injected with these transfectants fell severely sick and died by 21 d. In contrast, mice inoculated with F12-28 cells untransfected or transfected with the vector without the cDNA insert (Vec 3 and Vec 61 in Table II) developed tumors >20 d after injection and survived for as long as 50 d. Among the IL-6 cDNA transfectants, the latency period of tumor development apparently correlated with the level of IL-6 secreted by individual clones: a higher producer of IL-6 gave rise to a tumor in a shorter period. Tumorigenicity of the transfectants in syngeneic BALB/c mice was comparable with that in nude mice, that is, the IL-6 cDNA transfectants formed tumors and killed animals in a much shorter period than the mock transfectants, and the inverse correlation was observed between the latency period and the level of IL-6 production (Table II).

Discussion

The concept of autocrine stimulation of cell proliferation postulates that cells could escape from normal growth control and become malignant by the endogenous production of growth factors that act on themselves via functional external receptors (30, 31). The autocrine action of growth factors such as transforming growth factor and platelet-derived growth factor has been demonstrated in certain virus-transformed cells (32-35). There is ample evidence showing that autocrine self-stimulation could be involved in malignant transformation of lymphoid cells (21, 36-40). We have recently demonstrated by means of transfection and expression of an IL-2 gene in
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Syngeneic BALB/c and BALB/c nu/nu mice were inoculated subcutaneously with 10^5 cells from individual cell lines. The animals were monitored for the formation of tumors ≥5 mm in diameter. Results are expressed as the fraction number of mice with tumor per number of mice injected, where we put in the number of mice having tumors even if they were dead when monitored.
IL-2-dependent Th cells that the aberrant activation of the IL-2 autocrine circuit could lead T cells to malignant transformation, thus providing a model system for the understanding of the pathogenesis of adult T cell leukemia caused by infection of CD4⁺ Th cells with human T cell leukemia virus type 1 (23).

In the present study, we experimentally created cell lines that proliferated in vitro autonomously through an IL-6 autocrine mechanism by introducing the IL-6 cDNA expression vector into an IL-6-dependent B cell line. The autocrine nature of the proliferation is warranted by the following evidence: whereas F12-28 cells untransfected or transfected with the vector alone required exogenous IL-6 for survival and proliferation, the IL-6 cDNA transfectants did not. The transfectants secreted biologically active IL-6 into the culture media. Further, their proliferation was inhibited by antibodies to IL-6. The growth of the transfectants was a function of the cell density.

The establishment of cell lines that proliferated autonomously through IL-6 autocrine self stimulation made it possible to examine the relationship between IL-6 autocrine mechanism and tumorigenicity. The IL-6 cDNA transfectants were found to be highly tumorigenic when inoculated into syngeneic as well as athymic mice (Table II). The latency period of tumor development correlated apparently with the level of IL-6 secreted by individual clones; that is, the transfectants secreting a higher titer of IL-6 developed tumors in shorter periods. This fact indicated that autogenous IL-6 contributed to the tumorigenicity of the transfectants. Cells obtained from tumors proliferated in vitro in an IL-6 autocrine fashion indistinguishable from that of the inoculated cells (data not shown), discounting the possibility that IL-6 cDNA transfectants could become malignant in vivo by a mutation(s) that abrogated the requirement for IL-6 autocrine self stimulation.

While F12-28 cells transfected with the expression vector alone were absolutely dependent on exogenous IL-6 for survival and proliferation in vitro (Fig. 2 B), they produced tumors in vivo even after a much longer latency period than the IL-6 cDNA transfectants did (Table II). This cannot be due to the introduced expression vector or the consequence of DNA transfection, because untransfected F12-28 cells gave rise to tumors with a latency period comparable with that of mock transfectants. The in vivo growth of the IL-6-dependent cells might be explained by the paracrine action of a growth factor(s) such as IL-6 produced by cells in the host animals. Whereas tumors developed in mice injected with the IL-6 cDNA transfectants were almost exclusively made up of transfectants themselves, those in mice injected with F12-28 cells contained not only the inoculated cells but also numerous host cells, including fibroblasts, lymphocytes, and macrophages, which are potent producers of IL-6 (unpublished observation). Furthermore, cells of tumors produced by F12-28 cells retained the IL-6 dependence for their in vitro growth. These observations suggest a paracrine growth of factor-dependent cells in vivo. Alternatively, F12-28 cells might have already undergone some genetic alterations toward the acquisition of malignancy like other immortalized cell lines, and could grow in vivo without any requirement of IL-6, even though they are strictly dependent on IL-6 in vitro. Whatever the mechanism of in vivo growth of IL-6-dependent cells is, our results indicate that the acquisition of the ability to produce IL-6 greatly potentiates the tumorigenicity.

Tumors produced by the transfectants secreting large amounts of IL-6 were so malignant that host animals fell severely sick and died by 21 d after inoculation with 10⁵ cells. The high degree of malignancy did not seem to be attributed only to the
growing tumors in themselves, because the recipient mice died even before tumors became large, in contrast to mice injected with mock transfectants. Blood taken from the mice bearing tumors of the IL-6 cDNA transfectants was very viscous and contained an elevated level of acute phase proteins, such as α2-macroglobulin along with the reciprocal reduction of albumin detected by immunoelectrophoresis (unpublished observation). In the sera of the mice, 10^3 to 4 × 10^5 U/ml of IL-6 activity was detected, while <10 U/ml was detected in those of mice injected with the mock transfectants. These observations suggested that IL-6 produced by tumors had undesirable effects on host animals. In humans, such systemic effects of IL-6 have been observed in patients with cardiac myxomas producing IL-6 (41).

Kawano et al. (21) have reported that myeloma cells freshly isolated from patients secreted biologically active IL-6 and expressed its receptors. In about half of the cases, myeloma cells showed responsiveness to IL-6, and their proliferation in vitro was inhibited by anti-IL-6 antibodies. These growth properties of human myeloma cells are similar to those of our transfectants, although the levels of IL-6 produced by human myeloma cells were low as compared with those produced by the IL-6 cDNA transfectants. On the other hand, Klein et al. (42) have shown that the IL-6 production found in the bone marrows of patients with multiple myeloma was assigned to adherent cells of the bone marrow environment but not to myeloma cells, suggesting a paracrine rather than autocrine growth of myeloma cells, as observed in pristane-induced murine plasmacytomas (17–19). Since our results provide evidence that the establishment of IL-6 autocrine loop enhances the tumorigenicity of IL-6-dependent cells, it is possible to assume that myeloma cells proliferate through a paracrine mechanism in an early stage of oncogenesis and step up into a more advanced stage by acquiring the ability to produce IL-6. They might become independent of IL-6 autocrine self stimulation in a final stage, as myeloma cells from patients with stage III of multiple myeloma showed a poorer or no response to IL-6 (43). Taken together, we conclude that the dysregulated expression of IL-6 and its receptors confers on B cells a strong selective growth advantage in an autocrine fashion and results in the induction and/or progression of the malignant state of B cells.

Summary

We introduced an IL-6 cDNA expression vector into a murine B cell line, the growth of which definitely required the presence of exogenous IL-6. The transfected cells secreted substantial amounts of IL-6, to which they themselves responded by proliferating without further requirement of exogenous IL-6. The proliferation was a direct function of cell density and was inhibitable by antibodies to IL-6, indicating the autocrine nature of the growth. The IL-6 cDNA-transfected cells displayed greatly enhanced tumorigenicity when inoculated into syngeneic and nude mice. Our data suggest that an IL-6 autocrine self stimulation confers on B cells a selective growth advantage and results in the induction or progression of the malignant state of B cells.

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cutaneous T cell lymphoma and leukemia cell lines produce and respond to T cell growth factor. *J. Exp. Med.* 154:1403.


