bacterial species, including *Klebsiella, Shigella, Salmonella, Yersinia* and *Campylobacter*, all suspected of being involved in human arthritis[18]. Possibly humans could be exposed to the 180–188 epitope, or to a cross-reactive epitope associated with different environmental bacteria under conditions which might influence their susceptibility to autoimmune arthritis. If the 180–188 epitope is involved in the pathophysiology of arthritis, a purified peptide containing the epitope might induce therapeutic suppression of the disease process in the same way

as the 65K antigen in AA.

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Basic fibroblast growth factor fused to a signal peptide transforms cells

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Basic fibroblast growth factor (bFGF) is a potent growth and angiogenic factor that is found in abundance in tissues such as brain, hypothalamus, kidney and cartilage[1,2]. Despite this copious production of bFGF, most of these tissues are not undergoing either active growth or angiogenesis, suggesting that bFGF activity must be regulated so as to prevent autostimulation of cell growth. In cultured cells, bFGF is associated mainly with cells and basement membranes and is not released into the medium[3,4]. Prevention of release could be a mechanism for regulation of bFGF activity and may be a consequence of the apparent absence of a secretory-signal sequence in the bFGF protein[5]. Here we investigate whether this regulation can be overridden through the forced secretion of bFGF. Such secretion might provide the bFGF access to its receptor and in turn lead to autocrine transformation of the cell. We report that bFGF, as specified by a recombinant plasmid, is itself unable to induce such transformation, but acquires this ability after fusion with a secretory-signal sequence. The resulting transformants undergo unusual morphological alteration and display tumorigenicity.

We inserted a complementary DNA encoding bovine bFGF (ref. 5) into the pJay3 plasmid (J. Morgenstern, unpublished) expression vector (pbFGF, Fig. 1*a*). In a second clone, sequences specifying an amino-terminal immunoglobulin signal peptide of 19 amino acids, which mediates co-translational insertion of a nascent protein into endoplasmic reticulum, were fused to the bFGF cDNA (refs 6, 7). The fusion joins this signal peptide to the second amino-acid residue of the native bFGF (pIgbFGF; Fig. 1*b*). The amino terminus of the predicted primary translation product is illustrated in Fig. 1*c*. After translation this chimaeric protein should be inserted into the endoplasmic reticulum and the signal peptide cleaved off, resulting in a protein of relative molecular mass (M_r) 18,000 (18K) which is virtually indistinguishable in size from normal bFGF.

To ascertain the transforming potential of these expression clones, the plasmids were transfected into NIH 3T3 cells[8]. This cell line is known to express bFGF receptors and respond
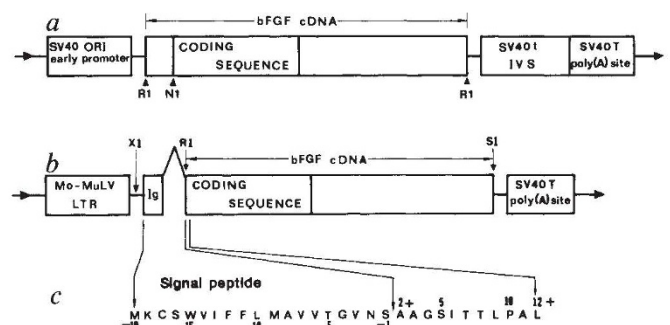

Fig. 1 Basic FGF expression constructs (R1, *Eco*R1; N1, *Nco*1; X1, *Xba*1; S1, *Sal*1 restriction site). *a*, pbFGF plasmid: the SV40 early promoter drives constitutive expression of the native bovine brain bFGF. *b*, pIgbFGF plasmid: a murine leukaemia virus long terminal repeat (LTR) containing promoter/enhancer sequences drives constitutive expression of the immunoglobulin signal peptide-bFGF fusion protein. *c*, Amino terminus of the predicted primary translation product of the chimaeric IgbFGF protein. The 19-amino-acid signal peptide is fused to the second amino acid of the native bovine brain bFGF.
Methods. The pbFGF plasmid was constructed by inserting the *Eco*RI-flanked bFGF cDNA sequence from pJJ11-1 plasmid[5] in the 5' to 3' orientation behind the SV40 early promoter sequences into the *Eco*RI site of the pJay3 mammalian expression vector. The pIgbFGF expression plasmid was constructed as follows. The pJJ11-1 plasmid was digested with *Nco*I restriction enzyme to remove all of the 5'-end and some of the 3'-end non-coding sequences. The resulting 1,040 base pair (bp) cDNA fragment was blunted with mung-bean nuclease and ligated to 8-mer *Eco*RI linkers. After digestion with *Eco*RI, the fragment was redigested with *Ssp*I to further remove ~430 bp of 3'-end non-coding sequence. The isolated 606-bp fragment was first ligated to *Sal*I linkers and then digested with *Sal*I restriction enzyme. The purified 610-bp fragment was ligated into the pUCDS3 vector[8] predigested with *Eco*RI and *Sal*I and with the synthetic EGF sequences removed.

mitotically to bFGF treatment (refs 3, 4 and unpublished observations). The pbFGF plasmid did not induce any foci of transformants, even though these cultures were observed for four weeks. A control plasmid (pUCDS5) containing only the immunoglobulin signal sequence[7] was also unable to induce focus formation. The pIgbFGF plasmid, however, induced foci with distinctive morphology. These foci were visible to the naked eye within 10 days of transfection, but were present only at a low frequency of ~40 foci per 8 μg DNA per 10^6 cells; but after two more weeks in culture, a second wave of foci appeared that

Fig. 2 Analysis of bFGF biosynthesis in monoclonal cell lines expressing bFGF and IgbFGF by heparin-affinity chromatography and immunoprecipitation. The three panels on the left show heparin-affinity chromatography elution profiles. Open circles represent cell lysates, closed circles represent conditioned media. The three panels on the right show the immunoprecipitations of cell lysates. Arrows show the positions of bFGF. *a*, NIH-NM2 control cells. Lane 1: NIH-NM2; lane 2: NIH-BNM7; lane 3: NIH-NM1 (another NIH 3T3 neomycin-resistant control cell line); lane 4: molecular weight markers. *b*, NIH-BNM7 cell line expressing bFGF. Lane 1: peptide control (immunoprecipitate of NIH-BNM7 in presence of excess peptide representing amino acids 33-43 of bFGF); lane 2: NIH-BNM7; lane 3: bovine aortic endothelial cells (BAE) (ref. 4); lane 4: molecular weight markers. *c*, NIH-IgBNM6-1 cell line expressing IgbFGF. Lane 1: NIH-IgBNM6-1 peptide control (this cell line immunoprecipitated in the presence of excess peptide as above); lane 2: NIH-IgBNM6-1; lane 3: molecular weight markers.

Methods. NIH 3T3 cells transfected with pSV2-neo alone (NIH-NM2), with pbFGF and pSV2-neo (NIH-BNM7), and with pIgbFGF and pSV2-neo (NIH-IgBNM6-1) were grown in culture and bFGF synthesis was assayed by immunoprecipitation and by heparin-Sepharose affinity chromatography. NIH-NM2, NIH-BNM7, and NIH-IgBNM6-1 cells produced $0.04+/-0.02$, $0.73+/-0.23$, $0.8+/-0.35$ units of cell-associated growth factor activity per 10^4 cells respectively, compared to $0.89+/-0.26$ units per 10^4 cells synthesized by bovine endothelial cells[4]. Immunoprecipitation of bFGF was carried out as follows. Cells were grown in DMEM (Gibco) supplemented with 10% calf serum, penicillin and streptomycin to a density of $\sim 3 \times 10^6$ cells per 100 mm dish. The cells were radiolabelled for 16 h using 5 ml of cysteine-free DMEM media (Gibco) supplemented with 5% calf serum, penicillin and streptomycin and 50 μCi of [^{35}S]cysteine (specific activity $> 1,000$ Ci mmol^{-1}, New England Nuclear) per 100 mm dish. After removal of media, the cells were washed twice with PBS and scraped into 1 ml cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$ and 0.5% (v/v) Nonidet P40 detergent). Following careful resuspension and a 5 min incubation on ice, the nuclei were pelleted with a 5 min centrifugation in an Eppendorf microfuge. The supernatant was pre-adsorbed with 5 μl normal rabbit serum and 40 μl protein A-Sepharose for 4 h at 4 °C. The protein A-Sepharose was removed by a 1 min microfuge centrifugation. The supernatant was incubated for 16 h at 4 °C with 5 μl of anti-amino-terminus peptide rabbit antibody[3,4]. As a control, 5 μg of immunizing peptide were also added to an identical sample. Twenty μl protein A-Sepharose was added for one hour of further incubation. The pellet was washed four times with cell lysis buffer with 1% Triton X-100, 1% sodium deoxycholate and 1% aprotinin solution (Sigma). Twenty μl SDS-PAGE sample buffer was added to the washed pellet and the sample electrophoresed on SDS-PAGE (ref. 22). Heparin Sepharose affinity chromatography was carried out as described[3,4]. Cells were grown for 5-6 days until they reached confluence. Conditioned medium (100 ml) was removed, and cells (0.5-1.5×10^8) were resuspended at 10^7 cells per ml in 10 mM Tris-HCl, pH 7, 1 M NaCl, and lysed by sonication. Cell lysates diluted to 0.1 M NaCl with conditioned medium were applied to individual heparin-Sepharose columns (3 ml) and bFGF was eluted with an 80 ml gradient of 0.1 M-3 M NaCl gradient at a flow rate of 30-40 ml h^{-1}. Fractions (4 ml) were collected and tested for ability to stimulate DNA synthesis in BALB-c/3T3 cells[3,4].
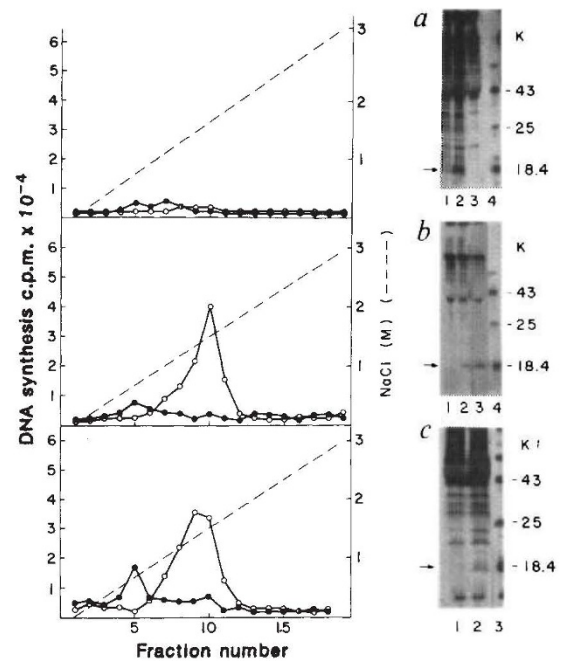
was 20-fold greater in number. These delayed foci did not arise through seeding of cells from the foci formed initially because they were also seen when the monolayer was overlaid with soft agar immediately after transfection.

Clonal cell lines synthesizing bFGF and IgbFGF proteins were derived by co-transfecting the expression vectors with the dominant selectable marker pSV2-neo (ref. 9). Toxicity of the pIgbFGF clone in these cotransfections was measurable; addition of 8 μg pIgbFGF to 0.1 μg transfected pSV2-neo DNA resulted in a tenfold reduction of G418-resistant colonies. A similar inhibitory effect has been described for the Abelson murine leukaemia virus transforming gene[10]. Possibly this could account for the extremely low focus-forming efficiency and the dramatic reduction in the number of neomycin-resistant colonies appearing after co-transfection with pIgbFGF.

Neomycin-resistant colonies were expanded into cell lines and screened for bFGF activity increased over control NIH 3T3 cells transfected with pSV2-neo alone. Figure 2 shows the analyses of three selected cell lines: a neomycin-resistant control NIH 3T3 cell line, NIH-NM2; a bFGF-expressing cell line, NIH-BNM7; and an IgbFGF-expressing cell line, NIH-IgBNM6-1. Basic FGF biosynthesis in these cell lines was analysed by immunoprecipitation of cell lysates and by heparin affinity chromatography of both cell lysates and conditioned medium[3,4].

Control NIH 3T3 cells did not synthesize detectable amounts of bFGF (Fig. 2a), but both NIH-BNM7 cells (Fig. 2b) and NIH-IgBNM6-1 cells (Fig. 2c) synthesized immunoprecipitable bFGF (Fig. 2, right) and biologically active, cell-associated bFGF that eluted from heparin-Sepharose columns at 1.5 M NaCl. The immunoprecipitable bFGF could be displaced

by an excess of the immunizing peptide used to induce anti-bFGF antibody. This material co-migrated with endothelial bFGF of $M_r \sim 18$K (ref. 4), indicating that the IgbFGF signal peptide was indeed cleaved off (Fig. 2c). The additional proteins of ~ 19K and 23K immunoprecipitated from the NIH-IgBNM6-1 cells could represent post-translationally modified bFGF, suggesting passage through the endoplasmic reticulum and the Golgi. In both cell types, bFGF was found associated with the cells rather than with the conditioned medium. No immunoprecipitable or heparin-binding bFGF was found in the conditioned medium of NIH-IgBNM6-1 cells. Culture of bovine aortic endothelial cells in the presence of the NIH-IgBNM6-1 conditioned media, or even co-culture of these two cell types, did not result in an accelerated proliferation of the endothelial cells.

The growth patterns of NIH-NM2, NIH-BNM7 and NIH-IgBNM6-1 cells were compared (Fig. 3). NIH-BNM7 cells (Fig. 3b) grew minimally in soft agar. Their morphology was not very different from the control cells (Fig. 3a), although some changes were noted: the growth rate in 2% serum was roughly doubled, and the saturation density was about fourfold that of NIH-NM2 control cells.

The morphology of NIH-IgBNM6-1 cells differed dramatically from both the control NIH-NM2 cells and the NIH-BNM7 cells and was identical to cells in pIgbFGF-induced foci (Fig. 3c). The NIH-IgBNM6-1 cells grew as large stellate aggregates that barely adhered to the dish. Despite their active growth in semisuspension in media, growth in soft agar was minimal.

Cell lines expressing IgbFGF were highly tumorigenic in syngeneic NIH/NSF mice, producing rapidly growing tumours within a week in all 50 mice tested. In contrast, no tumours
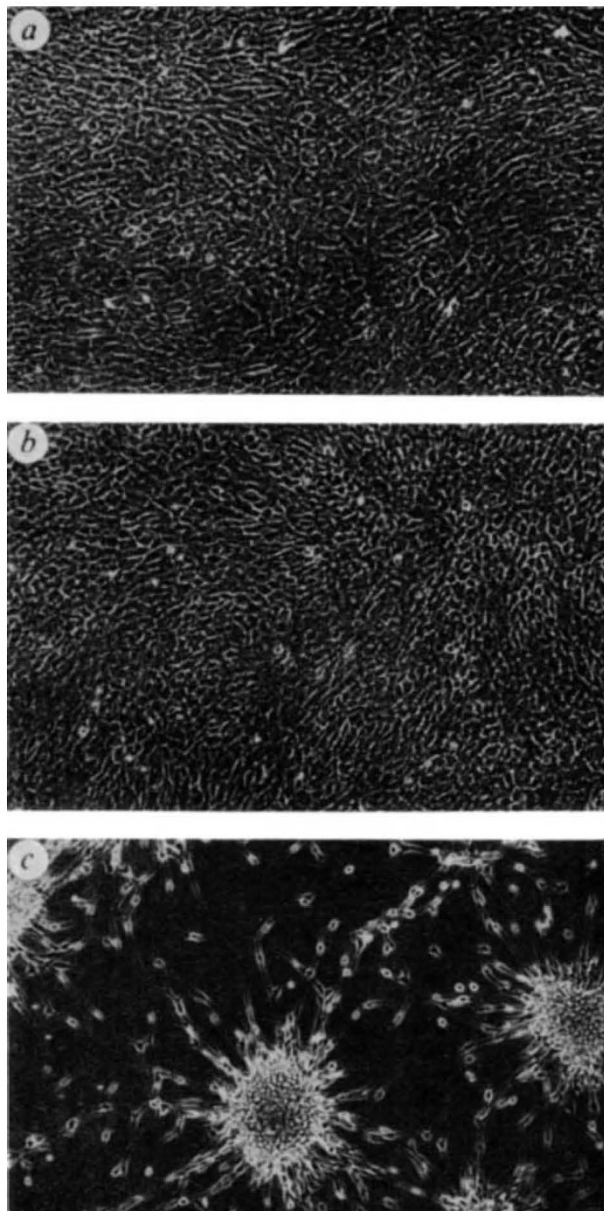
Fig. 3 Photomicrographs of neomycin-resistant monoclonal cell lines of NIH 3T3 cells transfected with a, pSV2-neo alone (NIH-NM2); b, pSV2-neo with pbFGF (NIH-BNM7); c, pSV2-neo with pIgbFGF (NIH-IgBNM6-1). Cells were grown in 100 mm Costar dishes and photographed with technical PAN 2415 film using a Nikon inverted microscope.

We cannot explain the delayed appearance of foci of transformants which occurs at a low, but predictable, frequency in virtually all cell lines that have acquired pIgbFGF DNA. This event may represent a change in the expression of pIgbFGF DNA in these cells (for example, de-repression of the Mo-MuLV LTR promoter activity due to its demethylation), or may reflect a host-cell change that confers responsiveness on the acquired gene.

These results show that cells expressing comparable amounts of bFGF have a phenotype that depends on whether or not this protein has a signal peptide to allow entrance into the endoplasmic reticulum. Basic FGF lacking a signal peptide, as found in endothelial cells in culture, in tissues in vivo, and in the NIH-BNM cell lines, does not appear to trigger autocrine transformation of cells. In contrast, addition of the signal peptide to this growth factor has a profound effect on cellular phenotype and tumorigenic potential. The cell lines expressing IgbFGF are transformed and tumorigenic even though no bFGF is apparent in the medium. Since IgbFGF is found as a processed 18K protein, it has presumably undergone cleavage by signal peptidases responsible for the removal of signal sequence in the endoplasmic reticulum. It could be subsequently secreted and then immediately sequestered at the cell surface, activating a mitogenic pathway and completing the autocrine loop through an extracellular step. An alternative hypothesis put forward to explain transformation by the sis oncogene[11,12,13] is that the signal peptide associated with bFGF directs this protein into the same cellular compartments that are encountered by bFGF receptor molecules during their own post-translational maturation. This growth factor may then bind its receptor at an intracellular location. A mitogenic response and resulting autocrine transformation could therefore be triggered even before the complex reaches the plasma membrane.

The present results show that the gene for bFGF, like those of other growth factors[14-18], is a potential oncogene. Recent studies have shown that three different oncogenes encode FGF-homologous proteins: int-2, hst, and a bladder oncogene[19-21]. Whether these oncogenes produce biologically active, secreted bFGF-like growth factors has not yet been determined. The amino-termini of the proteins encoded by these oncogenes contain hydrophobic sequences that may serve as signal peptides. Cell lines expressing the chimaeric signal peptide bFGF could be used to investigate how bFGF and homologous genes act as oncogenes.

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were induced by control NIH 3T3 cells and only one tumour in 27 mice was induced by cells expressing bFGF. The NIH-IgBNM tumours could be cultured in the presence of neomycin and shown to produce substantial amounts of immunoprecipitable, heparin-binding, biologically active bFGF (data not shown), demonstrating that the tumours were derived from bFGF-producing cells.

More than 40 neomycin-resistant cell lines were also obtained by co-transfection of the selectable marker with pIgbFGF. Many of these expressed barely detectable bFGF activity and did not display altered morphology at the time of isolation. However, during the first few rounds of passage, foci with stable transformed morphology appeared in cultures grown from these lines and expressed more bFGF than the parental clonal line when expanded. This delayed focus formation is reproducible and occurs in 90% of the neomycin-resistant cell lines co-transfected with pIgbFGF.