Tumour necrosis factor α confers an invasive, transformed phenotype on mammary epithelial cells

Roberto Montesano[1],*, Priscilla Soulié[1], Johannes A. Eble[2] and Fabio Carrozzino[1]

[1]Department of Cell Physiology and Metabolism, University of Geneva Medical School, CH-1211 Geneva 4, Switzerland
[2]Institute of Physiological Chemistry and Pathobiochemistry, Münster University Hospital, Münster 48149, Germany
*Author for correspondence (e-mail: roberto.montesano@medecine.unige.ch)

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Summary

Although loss of cell-cell adhesion and gain of invasive properties play a crucial role in the malignant progression of epithelial tumours, the molecular signals that trigger these processes have not been fully elucidated. In light of the well-established relationship between chronic inflammation and cancer, we hypothesized that pro-inflammatory cytokines disrupt epithelial-cell adhesion and promote cell migration. To test this hypothesis, we used an in vitro model in which 31EG4-2A4 mouse mammary epithelial cells grown in a collagen gel form compact spheroidal colonies. Among the several cytokines examined, tumour necrosis factor α (TNF-α) caused a pronounced 3D scattering of preformed epithelial-cell colonies and induced 31EG4-2A4 cells grown on top of a collagen gel to invade the underlying matrix. In addition, TNF-α abolished contact-mediated inhibition of cell proliferation and stimulated cell growth both in the absence of exogenous mitogens and under anchorage-independent conditions. TNF-α induced the expression of matrix metalloproteinase 9 (MMP-9). Addition of the MMP inhibitor BB-94 abrogated TNF-α-induced 3D scattering. TNF-α also enhanced the attachment of 31EG4-2A4 cells to type-I collagen and markedly increased the expression of the α2 integrin subunit. Addition of a blocking antibody to β1-integrin or of rhodocetin (a specific α2β1 antagonist) to collagen-gel cultures abrogated 3D scattering. Collectively, these results demonstrate an essential role for MMPs and α2β1 integrin in the invasive response of 31EG4-2A4 cells to TNF-α. We propose that the biological activities described in this study contribute to the ability of TNF-α to promote tumour progression and cancer-cell dissemination.

Key words: Cytokine, Migration, Invasion, Transformation, Integrin

Introduction

Epithelial tissues are composed of tightly connected cells, which, under physiological conditions, adopt a sedentary phenotype. However, in specific developmental settings, epithelial cells can transiently lose cell-cell adhesion and exhibit a migratory behaviour (Gumbiner, 1996; Hay, 1995). A similar phenotypic switch can inappropriately occur in post-natal life during carcinogenesis. Indeed, breakdown of intercellular contacts and gain of invasive properties are a hallmark of malignancy (Birchmeier et al., 1996; Mareel and Leroy, 2003; Thiery, 2002). Elucidating the molecular mechanisms responsible for loss of cell adhesion and acquisition of motility is therefore a prerequisite for the development of therapeutic strategies aimed at stabilizing epithelial-tissue architecture and preventing invasion (Mareel et al., 1993).

Reduced cell-cell adhesion and increased cell motility during carcinogenesis have been attributed largely to the activation of oncogenes and/or inactivation of tumour-suppressor genes within the malignant cells themselves. However, epithelial tumours are surrounded and infiltrated by untransformed host cells (e.g. fibroblasts, endothelial cells and leukocytes), and it is increasingly recognized that this stromal microenvironment profoundly influences tumour development (Bhowmick et al., 2004; Bissell and Radisky, 2001; De Wever and Mareel, 2003; Elenbaas and Weinberg, 2001; Liotta and Kohn, 2001; Maffini et al., 2004; Tlsty, 2001; Unger and Weaver, 2003). In particular, a growing body of experimental and clinical evidence indicates that inflammatory cytokines produced by tumour-associated leukocytes can significantly contribute to malignant progression (Balkwill and Mantovani, 2001; Brigati et al., 2002; Coussens and Werb, 2002; Dranoff, 2004; Pollard, 2004; Wilson and Balkwill, 2002).

TNF-α is a potent proinflammatory cytokine produced by many cell types, including macrophages, lymphocytes, endothelial cells and fibroblasts. In addition to acting as a key mediator of inflammation, TNF-α elicits pleiotropic effects in a wide range of cells by binding and activating two cell-surface receptors, designated TNF-R1 and TNF-R2. TNF-R1 is constitutively expressed in virtually all cell types and mediates most biological effects of TNF-α. By contrast, the expression of TNF-R2 is largely restricted to lymphoid and endothelial cells, and it mediates a limited number of TNF-α activities. TNF-α-mediated receptor trimerization causes the recruitment of several adaptor proteins, which activate multiple intracellular signalling pathways (Aggarwal, 2003; Baud and Karin, 2001; Dempsey et al., 2003; Locksley et al., 2001; MacEwan, 2002; Varfolomeev and Ashkenazi, 2004).

The role of TNF-α in tumour growth and dissemination is complex and apparently paradoxical. As implied in its name, TNF-α was originally identified by its ability to induce the necrosis of transplanted tumours in mice (Carswell et al., 1975;

Old, 1985). Subsequent clinical trials have shown that, when administered locally at high concentration, TNF-α exerts powerful anticancer activity by causing selective damage and obliteration of intratumoral blood vessels (Lejeune et al., 1998). However, several lines of evidence indicate that TNF-α can enhance the capacity of tumour cells to metastasize (Moore et al., 1999; Orosz et al., 1993). TNF-α is thought to exert its tumour-promoting activity through multiple cellular mechanisms that have only recently begun to be elucidated (Arnott et al., 2002; Szlosarek and Balkwill, 2003).

In attempting to understand better the factors responsible for the disruption of normal epithelial tissue organization during tumour progression, we set out to assess the potential effects of proinflammatory cytokines on the behaviour of mammary epithelial cells grown in collagen gels. In this study, we report that, of the several cytokines examined, TNF-α causes multicellular colonies of mammary epithelial cells to disaggregate and induces cells grown on top of a collagen gel to invade the underlying matrix. Moreover, we show that TNF-α confers on mammary epithelial cells several additional properties that are characteristic of malignantly transformed cells, including proliferation in the absence of exogenously added growth factors, anchorage-independent growth and loss of contact-mediated inhibition of proliferation. Mechanistically, by focusing on the induction of 3D scattering, we present evidence that this activity of TNF-α requires the activity of matrix metalloproteinases (MMPs) and is mediated by upregulation of α2β1 integrin.

Materials and Methods

Reagents

Recombinant murine TNF-α, interleukin-1α (IL-1α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), as well as human interleukin-8 (IL-8) and human epidermal growth factor (EGF) were purchased from PeproTech (London, UK). Transforming growth factor α (TGF-α) and human platelet TGF-β1 were purchased from Bachem (Bubendorf, Switzerland) and R&D Systems (Minneapolis, MN, USA), respectively. Hepatocyte growth factor (HGF) and platelet-derived growth factor AA (PDGF-AA) were gifts from R. Schwall (Genentech, San Francisco, CA, USA) and G. Gabbiani (University of Geneva, Switzerland), respectively. The synthetic MMP inhibitor BB94 and the related inactive isomer BB1268 were kindly provided by P. Brown (British Biotech Pharmaceuticals, Oxford, UK). Hamster monoclonal anti-mouse-TNF-R1 neutralizing antibody (MAB430), goat TNF-R1 agonist antibody (AF225) and irrelevant goat IgGs (AB-108C) were purchased from R&D Systems. Hamster monoclonal anti-mouse-TNF-R2 neutralizing antibody (cat. no. 557533), function-blocking antibody against β1-integrin (Ha2/5, cat. no. 555002) and isotype-matched anti-trinitrophenol (TNP) IgM (cat. no. 553957) were purchased as azide- and endotoxin-free reagents from BD Biosciences Pharmingen (San Diego, CA, USA). Armenian hamster monoclonal IgG against mouse α2-integrin subunit (clone HMalpha2) was purchased from Chemicon International (Temecula, CA, USA). Rhodocetin was purified from *Calloselasma rhodostoma* venom as previously described (Eble et al., 2001).

Cells

31EG4 cells, a nontumourigenic murine mammary epithelial cell line (Zettl et al., 1992), were generously provided by G. L. Firestone (Berkeley, CA, USA). As the cells were morphologically heterogeneous, we cloned the original population by limiting dilution and selected for cells exhibiting an epithelial morphology in low-density cultures. The clone 2A4 was used throughout this study (for

the sake of simplicity, 31EG4-2A4 cells are referred to as 2A4 cells). The cells were routinely grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 medium (Gibco, Basel, Switzerland) supplemented with 5% foetal calf serum (FCS; Gibco) and used between passages 7 and 19.

3D scattering assay

2A4 cells were harvested by trypsinisation from confluent cultures, centrifuged and washed in serum-free DMEM-F12 medium. The cells were centrifuged once again and resuspended in a serum-free, chemically defined medium consisting of DMEM-F12, ITS+ Premix [6.25 µg ml^{-1} insulin, 6.25 µg ml^{-1} transferrin, 6.25 ng ml^{-1} selenious acid, 1.25 mg ml^{-1} bovine serum albumin (BSA) and 5.35 µg ml^{-1} linoleic acid; Becton Dickinson, Meylan, France], 1 ng ml^{-1} EGF and 10 ng ml^{-1} recombinant human fibroblast growth factor 2 (FGF-2; a gift from P. Sarmientos, Farmitalia Carlo Erba, Milan, Italy). This medium is hereafter referred to as 'defined medium'. 2A4 cells were mixed with a type-I-collagen solution prepared as described previously (Montesano et al., 1991b) to obtain a concentration of 3×10^4 cells ml^{-1}. Aliquots of the cell suspension (400 µl) were then dispensed into 16-mm wells of four-well plates (Nunc, Kampstrup, Roskilde, Denmark). After a 10-minute incubation at 37°C to allow collagen gelation, 500 µl defined medium were added above the gels. For the initial screening of cytokine effects, the cells were treated immediately with various concentrations of the indicated agents, whereas, in subsequent experiments, they were first grown in defined medium for 3 days to allow the formation of small colonies and then incubated for an additional 6 days with or without TNF-α. Media and treatments were renewed every 3 days. In some experiments designed to assess cell behaviour in more complex extracellular matrices (ECMs), either 10% (vol/vol) Matrigel (BD Biosciences, Bedford, MA, USA) or 100 µg ml^{-1} hyaluronic acid (Sigma, cat. no. H-1751) were incorporated into the collagen gels. For quantification of 3D scattering, five randomly selected fields (measuring 0.5 mm × 0.7 mm) per experimental condition in each of at least three separate experiments were photographed under bright-field illumination using the 20× objective of a Nikon Diaphot TMD inverted photomicroscope. Quantification was carried out on positive prints (250× final magnification) by counting the number of apparently single cells per photographic field. Data were expressed as mean number of cells ± s.e.m., and statistical significance was determined using the Student's unpaired *t* test.

Two-dimensional scattering assay

2A4 cells were seeded into 1.5% gelatine-coated 35-mm wells of a six-well plate in DMEM-F12 medium supplemented with ITS+ Premix at 1.5×10^5 cells per well and incubated for 2 days to allow the formation of compact cell islands, at which time increasing concentrations of TNF-α were added. After 24 hours and 48 hours of treatment, three randomly selected fields (measuring 0.5 mm × 0.7 mm) per experimental condition in each of at least three separate experiments were photographed under phase contrast using the 20× objective of a Nikon Diaphot TMD inverted photomicroscope. Quantification of scattering was carried out on positive prints (250× final magnification) by counting in each photographic field the cells that were completely isolated or showed only a minimal area of contact with their neighbours. For inhibition experiments, the inhibitors were added 2 hours before the addition of TNF-α, and scattering was quantified 48 hours later. Results represent the means of three independent experiments ± s.e.m. per condition. Mean values were compared using Student's unpaired *t* test.

Invasion assay

The invasive properties of control and TNF-α-treated 2A4 cells were

investigated by growing the cells to confluence on a 3D collagen gel and subsequently assessing their ability to penetrate into the underlying matrix. Because 2A4 cells grow very slowly on the surface of collagen gels, a 10 µl drop of a concentrated cell suspension (2×10^6 cells ml^{-1} in defined medium) was deposited onto a collagen gel cast in a 22-mm well. Following a 2-hour incubation at 37°C to allow cell attachment, 1 ml medium was gently layered onto the gel. 24 hours later, the medium was changed and the cells were grown for a further 5 days in the presence or absence of different concentrations of TNF-α. By markedly reducing the total number of cells per well, this procedure offered the additional advantage of preventing the acidification of culture medium that occurs in post-confluent cultures. For quantification of invasion, five randomly selected fields (0.5 mm × 0.7 mm) per experimental condition in each of at least three separate experiments were photographed under bright-field illumination with a 20× objective at a focal plane beneath the surface of the gel. Quantification was carried out on positive prints (250× final magnification) by counting the number of all cellular structures (i.e. apparently single cells and cell cords) identifiable in each photographic field.

Cell-proliferation assays

To assess whether TNF-α was able to stimulate cell proliferation under growth-factor deprivation, 2A4 cells were seeded into 16-mm wells at 1×10^4 cells per well in serum-free DMEM-F12 medium supplemented with ITS+ Premix. After 24 hours, the medium was removed, fresh medium was added and the cultures were incubated for an additional 9 days with various concentrations of TNF-α. Media and treatments were renewed every 2-3 days. To determine the time course of TNF-α-induced cell proliferation, cells were incubated for 4 days, 8 days, 12 days and 16 days with or without 10 ng ml^{-1} TNF-α. Cells in triplicate wells were harvested by trypsinization at the indicated time points and counted with a haemocytometer. Results represent the mean of three independent experiments ± s.e.m. per condition. Mean values were compared using Student's unpaired t test.

To assay anchorage-independent growth, 2A4 cells were suspended at a concentration of 3×10^4 cells ml^{-1} in defined medium in low-gelling-temperature agarose cast in 16-mm wells as described previously (Montesano et al., 1991b) and grown for 14 days in the presence or absence of TNF-α. For quantification, pictures of three randomly selected fields (1 mm × 1.4 mm) in each of three separate experiments were taken under bright-field illumination with a 10× objective. The number of colonies (arbitrarily defined as cell aggregates with a diameter greater than 40 µm) in each photographic field was assessed on positive prints.

To determine whether TNF-α treatment disrupts contact-mediated inhibition of cell proliferation, 6×10^4 cells were seeded in 20 µl defined medium within a glass cylinder (6 mm internal diameter) placed in the centre of a 22 mm well. After a 24 hour incubation period to allow the cells to attach and become confluent, the cylinder was removed and the cells were grown in 1 ml defined medium with or without TNF-α. Alternatively, a 10 µl drop of a concentrated cell suspension (2×10^6 cells ml^{-1}) was deposited in the centre of a 22-mm well. Following a 2-hour incubation, 1 ml medium was added. 24 hours later, the medium was changed and the cells were grown for a further 5-9 days in the presence or absence of TNF-α.

Gelatinolytic zymography

2A4 cells were plated in 60-mm plastic dishes in defined medium, grown to confluence and further incubated with or without 10 ng ml^{-1} TNF-α. After 48 hours, conditioned media were collected, supplemented with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 15 mM HEPES, and centrifuged at 340 g for 5 minutes. The resulting supernatants were stored at –20°C until use, then concentrated threefold by centrifugal filtration using a Centricon YM-

10 cartridge (Amicon-Millipore, Volketswil, Switzerland). Supernatants were mixed with SDS sample buffer without reducing agents and electrophoresed at 4°C in 7.5% SDS-polyacrylamide gels co-polymerized with 1 mg ml^{-1} gelatine. After soaking in 2.5% Triton X-100 for 20 minutes to remove SDS, the gels were incubated in reaction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl$_2$) at 37°C for 16 hours and then stained with an ethanol:acetic-acid:water mixture (30:10:60) containing 0.25% Coomassie Blue R250 for 4 hours. Conditioned media from MCF-7 breast-cancer cells and retinoic-acid-treated Eph4-J3B1A murine mammary epithelial cells (Montesano and Soulie, 2002), which are known to secrete MMP-2 and MMP-9, respectively, were used as migration standards. Gelatinolytic activity was detected as clear bands on a background of uniform blue staining.

Collagen-binding assay

Type-I collagen was extracted from rat-tail tendons as described previously (Montesano and Orci, 1985). Bacteriological 35-mm dishes (Falcon, cat. no. 351008) were incubated at 37°C with either type-I collagen diluted in double-distilled water to approximately 30 µg ml^{-1}, or a solution of 1.5% gelatine. After 30 minutes, the dishes were washed with PBS and incubated with 0.5% BSA in PBS for 30 minutes at 37°C to saturate nonspecific cell-binding sites. In control dishes, the collagen-coating step was omitted. 2A4 cells were harvested by trypsinisation from confluent cultures following a 48 hour preincubation in the presence or absence of 10 ng ml^{-1} TNF-α in defined medium, centrifuged and resuspended in serum-free DMEM-F12 medium supplemented with 0.5% BSA (DMEM-F12-BSA). The cells were then seeded into collagen- or gelatine-coated dishes at a concentration of 4×10^5 cells per dish. After a 60 minute incubation at 37°C, the medium was removed and unattached cells were gently washed away three times with DMEM-F12-BSA. The attached cells were then fixed in 2.5% glutaraldehyde in PBS. After washing in PBS, five randomly selected fields were photographed in each dish using a Nikon Diaphot TMD inverted photomicroscope and a 10× objective, and the number of attached cells per field was counted on positive prints (final magnification of 125×). Results represent the mean number of cells per field ± s.e.m. from three independent experiments. Mean values were compared using Student's unpaired t test.

Flow cytometry

Confluent monolayers of 2A4 cells in defined medium were treated with TNF-α (10 ng ml^{-1}) or left untreated. After 48 hours, the cells were rinsed twice with Ca^{2+}- and Mg^{2+}-free PBS (PBS$^{-/-}$) and harvested by trypsinization. Trypsin activity was inhibited by the addition of serum-supplemented medium. After washing in PBS$^{-/-}$, the cells were resuspended in PBS$^{-/-}$ containing 0.2% BSA to obtain a final cell concentration of 6×10^5 cells per sample and incubated for 45 minutes on ice with hamster monoclonal antibody against mouse α2-integrin subunit (5 µg ml^{-1}). After extensive washing with PBS$^{-/-}$, the cells were incubated on ice for 30 minutes in the dark with fluorescein isothiocyanate (FITC) labelled goat anti-hamster secondary antibody (1/300 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were washed three times with PBS$^{-/-}$ and α2-integrin expression on the cell surface was analysed by flow cytometry using a FACSFitc apparatus (Becton Dickinson) and CellQuest (Becton Dickinson)/WinMDI software (Scripps Research Institute, La Jolla, CA). Cells incubated with secondary antibody alone were used as negative controls. Mean fluorescence values obtained from five independent experiments were normalized to secondary antibody fluorescence. Mean values were compared using Student's unpaired t test.

Processing for light and electron microscopy

Two-dimensional cultures in plastic dishes were fixed with 2.5%

glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4), rinsed in cacodylate buffer and postfixed for 20 minutes in 1% osmium tetroxide in Veronal acetate buffer. They were then stained en bloc with 2.5% uranyl acetate in ethanol for 20 minutes, dehydrated in an ethanol series and embedded in Epon 812. To obtain sections perpendicular to the culture plane, the polymerized Epon disks were cut into 'matchsticks', which were then re-embedded in flat moulds as described previously (Montesano et al., 1984). Thin sections were cut with an LKB ultramicrotome (LKB Instruments, Gaithersburg, MD, USA), stained with uranyl acetate and lead citrate, and examined in a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

Results

TNF-α induces 3D and 2D scattering of 2A4 mammary epithelial cells

The objective of this study was to establish whether proinflammatory cytokines can disrupt epithelial-cell adhesion and promote cell migration in a 3D ECM environment. To this end, we set up an in vitro system in which 2A4 cells, a subclone of the spontaneously immortalized 31EG4 murine mammary epithelial cell line (Zettl et al., 1992), are grown in type-I-collagen gels in serum-free, chemically defined medium. Under these conditions, 2A4 cells formed compact spheroidal colonies within the collagen matrix (Fig. 1A). To assess the potential effect of proinflammatory cytokines on the behaviour of 2A4 cells, collagen-gel cultures were incubated with TNF-α, IL-1α, IL-1β, IL-6 or IL-8. These cytokines were chosen because they are key inflammatory mediators produced by tumour-associated macrophages (Balkwill and Mantovani, 2001; Leek and Harris, 2002; Pollard, 2004) and they were tested over a wide range of concentrations (100 pg ml^{-1} to 50 ng ml^{-1}). IL-8 had no discernible effect, whereas addition of relatively high concentrations (10-50 ng ml^{-1}) of IL-6 resulted in the formation of colonies with a range of morphologies, ranging from spheroidal clusters to loose cell associations (not shown). IL-1α and IL-1β had a more pronounced effect, because they induced the formation of loose cell clusters at concentrations higher than 500 pg ml^{-1} (Fig. 1B). The most dramatic changes, however, were observed following treatment with TNF-α (1-50 ng ml^{-1}), which resulted in the dispersion of single cells throughout the gel (Fig. 1C). These qualitative observations were confirmed by a quantitative analysis of 3D scattering in response to optimally effective concentrations (10 ng ml^{-1}) of IL-6, IL-1α, IL-1β and TNF-α (Fig. 1D). To determine whether other macrophage-derived growth or motility factors could induce 3D scattering, collagen-gel cultures of 2A4 cells were treated with either HGF (1-50 ng ml^{-1}), PDGF-AA (1-50 ng ml^{-1}), TGF-α (0.5-50 ng ml^{-1}) or TGF-β1 (0.1-10 ng ml^{-1}). HGF, PDGF-AA and TGF-α had no detectable effect on the organization of 2A4 cell colonies, whereas TGF-β1 induced a modest degree of cell scattering associated with a marked inhibition of cell proliferation in the gels at all concentrations tested (data not shown).

Having found that addition of TNF-α (and, to a lesser extent, of IL-1α or IL-1β) at the time of cell seeding induced 2A4 cells to disperse throughout the gel, we next asked whether these inflammatory cytokines could also be able to promote the disaggregation of established multicellular colonies. To address this question, 2A4 cells were grown for 3 days in a collagen gel to allow the formation of small spheroidal clusters,

at which time they were incubated with or without TNF-α, IL-1α or IL-1β for a further 6 days. When left untreated, the clusters progressively increased in diameter, eventually forming compact balls of tightly packed cells (Fig. 2A). Addition of IL-1α or IL-1β (1-10 ng ml^{-1}) induced slight alterations in colony morphology without overtly disrupting 3D organization (not shown). By contrast, addition of TNF-α (3-30 ng ml^{-1}) caused the dissociation of preformed cell clusters and the scattering of individual cells throughout the collagen matrix (Fig. 2B).

Although gels of type-I collagen provide a valuable in vitro model of the interstitial matrix within which invasive cells migrate during tumour progression, they only partially recapitulate the composition of the mammary-gland ECM. To determine whether 2A4 cells would behave differently in a more complex matrix environment, we suspended them in gels composed of either type-I collagen and Matrigel (a basement-membrane-like matrix) or type-I collagen and hyaluronic acid.
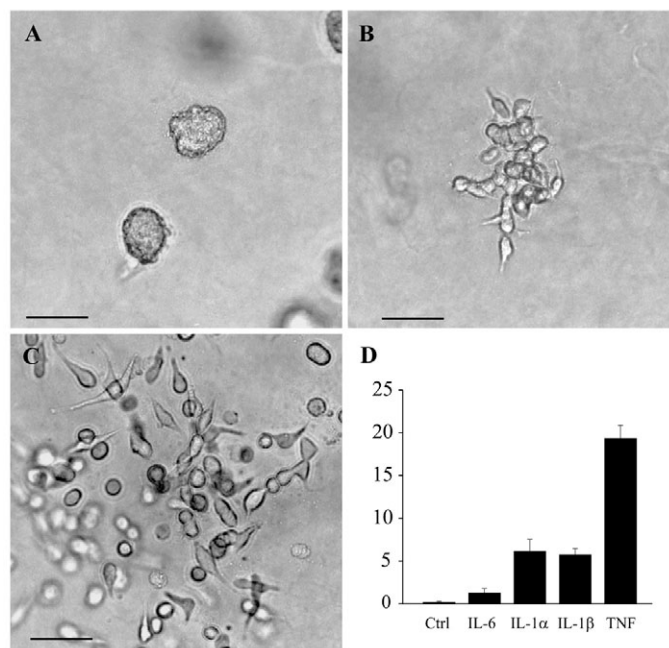
Fig. 1. Effect of proinflammatory cytokines on the behaviour of 2A4 mammary epithelial cells grown in collagen gels. The cells were suspended in a gelling collagen solution. After a 10 minute incubation at 37°C to allow collagen gelation, defined medium with or without the indicated cytokines was added above the gels and renewed after 3 days. (A) 2A4 cells grown in a collagen gel for 6 days under control conditions form compact ball-like colonies. (B) 2A4 cells treated for 6 days with IL-1β (2 ng ml^{-1}) form loose cell clusters. (C) 2A4 cells treated for 6 days with TNF-α (10 ng ml^{-1}) do not form cohesive colonies but scatter throughout the collagen matrix, mostly as individual cells. Scale bars, 50 μm. (D) Quantification of the effects of IL-6, IL-1α, IL-1β and TNF-α was carried out after 6 days of treatment by counting the number of apparently single cells in five randomly selected fields per experimental condition in each of three independent experiments. Data were expressed as mean number of cells ± s.e.m. and statistical significance was determined using the Student's unpaired t test. P=0.025 for values of IL-6 compared with controls; P<0.0005 for values of IL-1α, IL-1β and TNF-α compared with controls, as well as for values of TNF-α compared with IL-6, IL-1α, IL-1β.

In both types of multicomponent matrix, addition of TNF-α stimulated robust 3D scattering (data not shown), similar to what was observed in gels composed solely of type-I collagen. Based on these findings, we used collagen gels without additional ECM components for the remainder of this study. A quantitative analysis of TNF-α-induced 3D scattering in collagen gels demonstrated a maximal effect at 10-30 ng ml⁻¹ (Fig. 2G).

We next asked whether TNF-α-induced scattering required the interaction of 2A4 cells with a 3D collagen matrix or could also occur on a 2D plastic substrate. To address this question, 2A4 cells were plated in tissue-culture wells in minimally supplemented medium (DMEM-F12 with ITS+ Premix) and grown for 2 days to allow the formation of small islands of tightly packed cells. Thereafter, the cultures were incubated for 48 hours in the presence or absence of increasing

concentrations of TNF-α. Under these conditions, treatment with TNF-α caused an initial enlargement of cell colonies (not shown) followed by disruption of intercellular contacts and progressive cell dispersal (Fig. 2C,D). 2D scattering was reversed after TNF-α withdrawal and incubation in control medium for 72 hours (Fig. 2E,F). A quantitative analysis demonstrated a dose- and time-dependent induction of 2D scattering by TNF-α (Fig. 2H).

TNF-α induces monolayer-grown 2A4 cells to invade the underlying collagen matrix

To determine whether TNF-α-induced loss of cell-cell adhesion was accompanied by the acquisition of an invasive phenotype, we evaluated the ability of 2A4 cells grown atop of a 3D collagen gel to penetrate into the underlying matrix. The cells were plated onto a collagen gel, allowed to form a monolayer and subsequently incubated with various concentrations of TNF-α. Invasion was monitored by focusing beneath the surface of the gel. After 5 days, untreated cells displayed only a moderate degree of invasion into the underlying matrix (Fig. 3A). By contrast, TNF-α-treated cells extensively penetrated
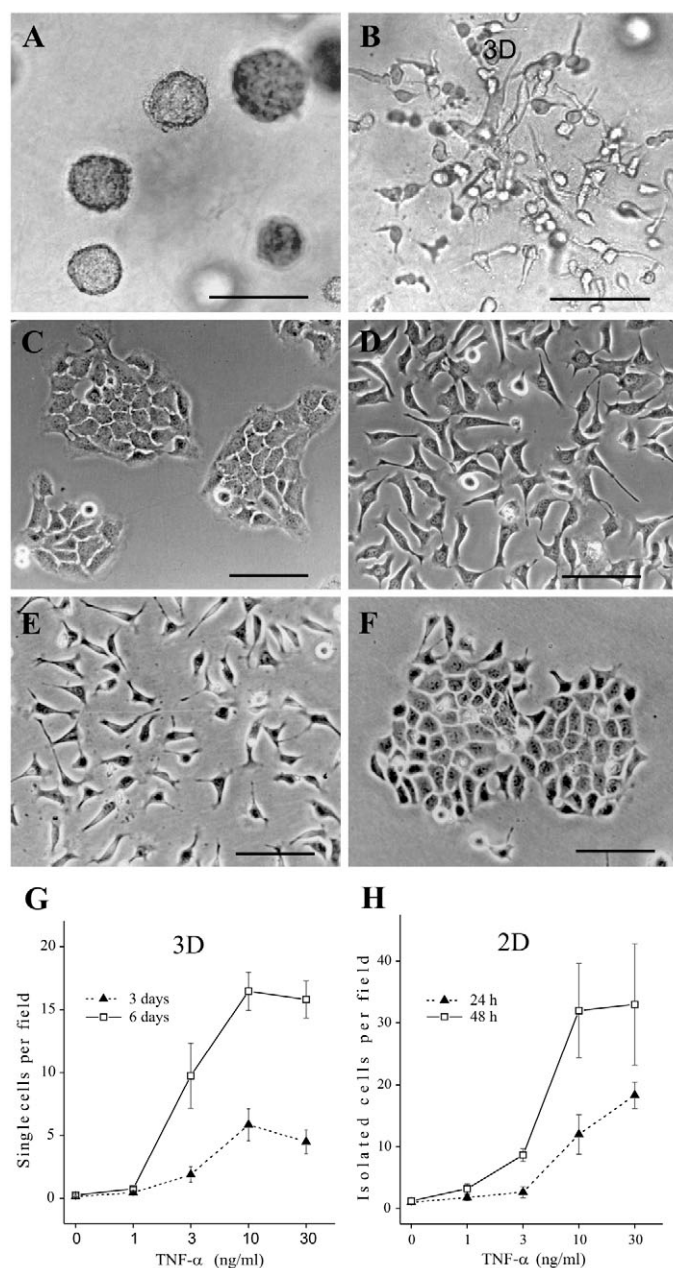
Fig. 2. TNF-α induces 3D and 2D scattering of 2A4 mammary epithelial cells. (A) 2A4 cells grown for 9 days in a collagen gel in defined medium have formed compact ball-like structures. (B) 2A4 cells were grown for 3 days in a collagen gel to allow the formation of spheroidal cell clusters, at which time 10 ng ml⁻¹ TNF-α was added for an additional 6 days. TNF-α treatment has resulted in the dissociation of preformed colonies and the dispersal of individual cells within the collagen matrix. (C) 2A4 cells seeded in plastic wells (2D culture) in DMEM-F12 with ITS+ Premix and grown for 4 days have formed discrete islands of closely apposed cells. (D) 2A4 cells were grown in DMEM-F12 with ITS+ Premix for 2 days to allow formation of small compact colonies before addition of TNF-α (10 ng ml⁻¹) for an additional 48 hours. TNF-α has induced a pronounced 2D scattering of 2A4 cells. (E) 2A4 cells maintained for 5 days in the presence of TNF-α are fully scattered. (F) Parallel cultures were treated 48 hours with TNF-α to induce cell dispersion, at which time the medium was changed and the cells were incubated for an additional 72 hours without TNF-α. The cells have re-established close intercellular contacts and formed compact epithelial-like colonies. (A-F) Scale bars, 100 μm. (G) Time course and dose dependence of TNF-α-induced 3D scattering. 2A4 cells were grown in collagen gels for 3 days and subsequently incubated for an additional 6 days with or without increasing concentrations of TNF-α. Quantification was carried out by counting the number of apparently single cells per photographic field after 3 days and 6 days of treatment. Data represent the mean number of single cells ± s.e.m. $P<0.05$ for values of 1 ng ml⁻¹ TNF-α compared with controls (3 days); $P<0.01$ for values of 1 ng ml⁻¹ TNF-α compared with controls (6 days); $P<0.005$ for values of 3 ng ml⁻¹ TNF-α compared with controls (3 days). (H) Time course and dose dependence of TNF-α-induced 2D scattering. 2A4 cells were grown for 2 days in gelatine-coated wells and subsequently incubated with or without increasing concentrations of TNF-α. Scattering was quantified by counting cells that were completely isolated or showed only a minimal area of contact with their neighbours. Data represent the mean number of isolated cells ± s.e.m. in nine randomly selected photographic fields. $P<0.05$ for values of 3 ng ml⁻¹ TNF-α compared with controls (24 hours); $P<0.0025$ for values of 10 ng ml⁻¹ TNF-α compared with controls (24 hours); $P<0.025$ for values of 1 ng ml⁻¹ TNF-α compared with controls (48 hours); $P<0.0005$ for values of 3 ng ml⁻¹ TNF-α compared with controls (48 hours). Scale bars, 100 μm.
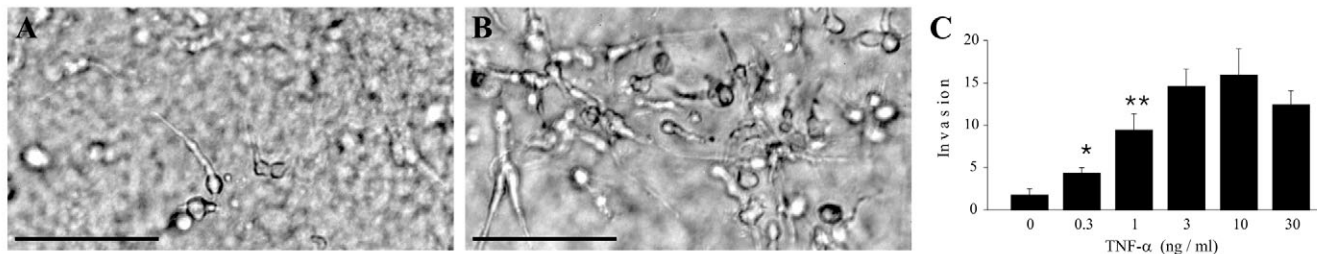
Fig. 3. TNF-α stimulates invasion of collagen gels. 2A4 cells were plated on top of a collagen gel, allowed to form a monolayer and subsequently incubated with or without different concentrations of TNF-α. 5 days later, the cultures were photographed at a focal plane below the surface to visualize cells that have penetrated the gel. In untreated cultures (A), only rare cells migrate from the surface monolayer into the underlying collagen matrix. By contrast, in cultures treated with TNF-α (3 ng ml^{-1}) (B), there is extensive invasion of the collagen gel. Scale bars, 100 μm. (C) Invasion was quantified by counting the number of all cellular structures (i.e. apparently single cells and cell cords) present in a photographic field at a focal plane beneath the surface monolayer. Five randomly selected fields per condition were analysed in each of three separate experiments. *$P<0.025$ (compared with control values); **$P<0.0005$ (compared with control values).

the collagen gel, either as single cells or as thin cell cords (Fig. 3B). A quantitative analysis demonstrated a dose-dependent stimulation of invasion, a maximal effect being observed with 10 ng ml^{-1} TNF-α (Fig. 3C).

TNF-α stimulates both anchorage-dependent and -independent growth of 2A4 cells

The finding that TNF-α induces 2D and 3D scattering of 2A4 cells and promotes their invasion into a collagen matrix prompted us to investigate whether TNF-α could also confer upon mammary epithelial cells additional properties that are characteristic of malignantly transformed cells. We first assessed the potential effect of TNF-α on cell proliferation under growth-factor deprivation. 2A4 cells were sparsely seeded in plastic wells in minimally supplemented medium (serum-free DMEM-F12 with ITS+ Premix). 24 hour later, they were either left untreated or treated with 10 ng ml^{-1} TNF-α. After 5 days, control cells had formed only small colonies (Fig. 4A), whereas TNF-α-treated cells had formed a nearly confluent monolayer (Fig. 4B). Determinations of cell number after 9 days of treatment with different concentrations of TNF-α demonstrated a robust dose-dependent stimulation of cell proliferation, a maximum effect being observed with 10 ng ml^{-1} TNF-α (Fig. 4C). To document the growth-stimulating effect of TNF-α in more detail, the proliferation of control cells and TNF-α-treated 2A4 cells was compared over a 16-day period. The results demonstrated sustained TNF-α-induced proliferation between 4 days and 12 days of treatment, after which a plateau was reached (Fig. 4D).

One of the fundamental attributes of malignantly transformed cells is anchorage-independent growth, which is exemplified by their ability to form multicellular colonies within non-adhesive 3D substrata such as soft agar or agarose. When grown in agarose gels under control conditions for 14 days, 51.7% of 2A4 cells did not proliferate, 44.6% formed small clusters of 2-10 cells and only 3.7% formed clusters of 11-24 cells, as determined by DAPI staining of cell nuclei (Fig. 4E and data not shown). By contrast, TNF-α-treated cells formed large colonies containing up to 64 cells (Fig. 4F and data not shown). A quantitative analysis demonstrated a dose-dependent induction of colony formation by TNF-α, with a maximal effect observed at 10 ng ml^{-1} (Fig. 4G).

TNF-α disrupts contact-mediated inhibition of cell proliferation

In the course of experiments aimed at assessing the growth-promoting activity of TNF-α in 2D cultures, we noticed that, in the presence of TNF-α, 2A4 cells continued to proliferate beyond confluence and tended to pile up, suggesting that they lost contact inhibition (i.e. cell-density-induced proliferation arrest). Initial attempts to analyse this phenomenon further in long-term cultures were, however, hampered by the deleterious effect of medium acidification on cell viability. To circumvent this problem, we generated a disk-shaped monolayer of 2A4 cells in the centre of a tissue-culture well by seeding a concentrated cell suspension within a glass cylinder. 24 hours later, the cultures were left untreated or were treated with TNF-α (1-10 ng ml^{-1}) for 5-9 days. Over this period, control cells exhibited strict contact inhibition and maintained typical cobblestone morphology (Fig. 5A,C). By sharp contrast, TNF-α-treated cells formed a network of thick anastomosing ridges lying on top of the existing monolayer (Fig. 5B). Cell multilayering in cultures of TNF-α-treated cells was confirmed by the examination of thin sections perpendicular to the bottom of the dishes (Fig. 5D). Similar results were obtained by seeding 2A4 cells at high density in a 10 μl drop of medium (data not shown).

TNF-α-induced cell scattering is mediated by TNF-R1

TNF-α exerts its biological effects by activating two cell-surface receptors, TNF-R1 and TNF-R2. To determine whether TNF-α-induced cell scattering is mediated by TNF-R1 or TNF-R2, 2A4 cells were seeded in collagen gels or in plastic dishes in defined medium and were subsequently incubated with TNF-α in the presence of blocking antibodies to either TNF-R1 or TNF-R2. Addition of antibodies to TNF-R1 suppressed TNF-α-induced 2D (Fig. 6A) as well as 3D (not shown) scattering. By contrast, blocking antibodies to TNF-R2 were not inhibitory (Fig. 6B). To substantiate these findings, we next used an anti-TNF-R1 antibody endowed with agonistic activity. When added to 2A4 cells suspended in collagen gels, the agonist anti-TNF-R1 antibody stimulated 3D scattering (Fig. 6D), whereas a control antibody had no effect (Fig. 6C). Taken together, these results indicate that TNF-α-induced scattering of 2A4 cells is mediated by TNF-R1.
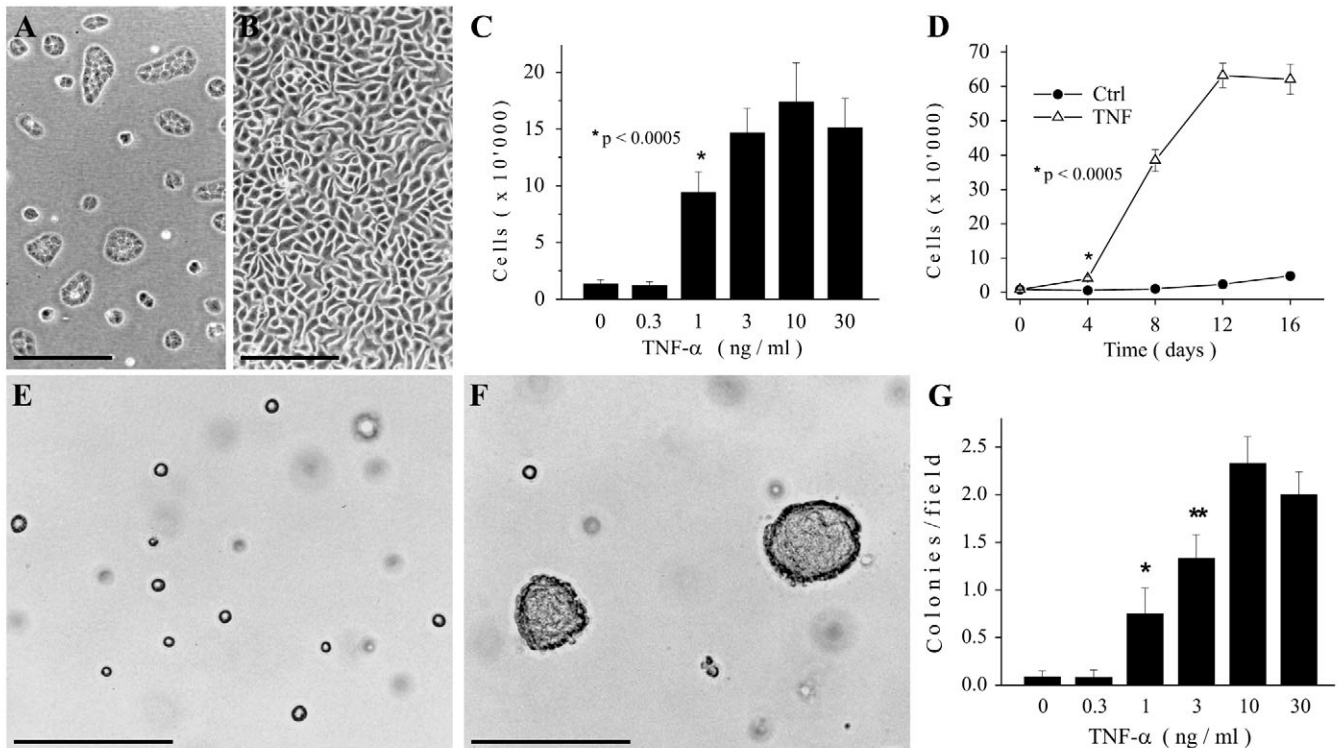
**Fig. 4.** TNF-α stimulates anchorage-dependent and -independent growth of 2A4 cells. (A,B) 35 mm tissue culture dishes were seeded with 2A4 cells at a density of 2×10^5 cells per dish in serum-free DMEM-F12 medium supplemented with ITS+ Premix. After 24 hours, the medium was renewed and the cultures were left untreated or were treated with 10 ng ml^{-1} TNF-α. 5 days later, control cells have formed only small colonies (A), whereas TNF-α-treated cells have formed a nearly confluent monolayer (B). Scale bars, 200 μm. (C) Dose dependence of TNF-α-induced cell proliferation. 2A4 cells were seeded in 16 mm wells at 1×10^4 cells per well in serum-free DMEM-F12 medium supplemented with ITS+ Premix. After 24 hours, the medium was removed, fresh medium was added, and the cultures were incubated with or without various concentrations of TNF-α. Medium and treatments were renewed every 2-3 days. Cells in triplicate wells were harvested by trypsinization after 9 days and counted with a haemocytometer. Data represent the means ± s.e.m. of at least three independent experiments. Mean values were compared using Student's unpaired *t* test. *$P<0.0005$ (compared with control values). (D) Time course of TNF-α-induced cell proliferation. 2A4 cells were seeded in 16 mm wells at a concentration of 1×10^4 cells per well in serum-free DMEM-F12 medium supplemented with ITS+ Premix. After 24 hours, the medium was changed and the cells were incubated for 4 days, 8 days, 12 days and 16 days with or without 10 ng ml^{-1} TNF-α. Cells in triplicate wells were harvested and counted at the indicated time points. Data represent the means ± s.e.m. of at least three independent experiments. Mean values were compared using Student's unpaired *t* test. *$P<0.0005$ (compared to controls at 4 days). (E,F) Assay of anchorage-independent growth. 2A4 cells were suspended at 3×10^4 cells ml^{-1} in agarose gels and grown for 14 days in the absence (E) or presence (F) of TNF-α. Scale bar, 200 μm. (G) Quantification of colony formation. Three randomly selected fields per experimental condition were photographed, and the number of colonies greater than 40 μm was determined in each photographic field. Data represent the means ± s.e.m. of at least three independent experiments. Mean values were compared using Student's unpaired *t* test. *$P<0.025$; **$P<0.001$ (compared with control values).

TNF-α-induced 3D scattering and invasion of collagen gels require metalloproteinase activity

The migration of cells through collagen matrices requires the activity of MMPs (Egeblad and Werb, 2002). To determine whether MMP activity was required for TNF-α-induced cell scattering in collagen gels, we examined the behaviour of 2A4 cells grown in the presence of TNF-α and different concentrations of the hydroxamate-based MMP inhibitor BB94 (Brown, 1998). We found that cell scattering was abrogated in a dose-dependent manner by BB94, but was not affected by the related inactive isomer BB1268 (Fig. 7A-C). Similarly, addition of BB94 suppressed the invasion of monolayer-grown 2A4 cells into the underlying collagen matrix in a dose-dependent manner (Fig. 7D). These findings imply that metalloproteinase activity is necessary for TNF-α-induced 3D scattering and collagen-gel invasion. To assess

whether TNF-α modulates the production or activity of MMPs involved in ECM degradation, we used gelatine-substrate zymography. Zymographs of conditioned media from untreated 2A4 cells inconstantly showed faint bands of gelatine lysis corresponding to the molecular weight of the latent form of MMP-2 (72 kDa gelatinase, gelatinase A), whereas proteolytic activity corresponding to the molecular weight of MMP-9 (92 kDa gelatinase, gelatinase B) was not detectable. The addition of TNF-α, however, induced the production of MMP-9 (Fig. 7E), but not of MMP-2, indicating a selective effect of TNF-α on MMP-9 production. Another MMP that plays an important role in collagen degradation is membrane type-1 MMP (MT1-MMP or MMP-14). To determine whether MT1-MMP is upregulated by TNF-α, total cellular RNA was analysed by northern-blot hybridization. We found that MMP-14 is
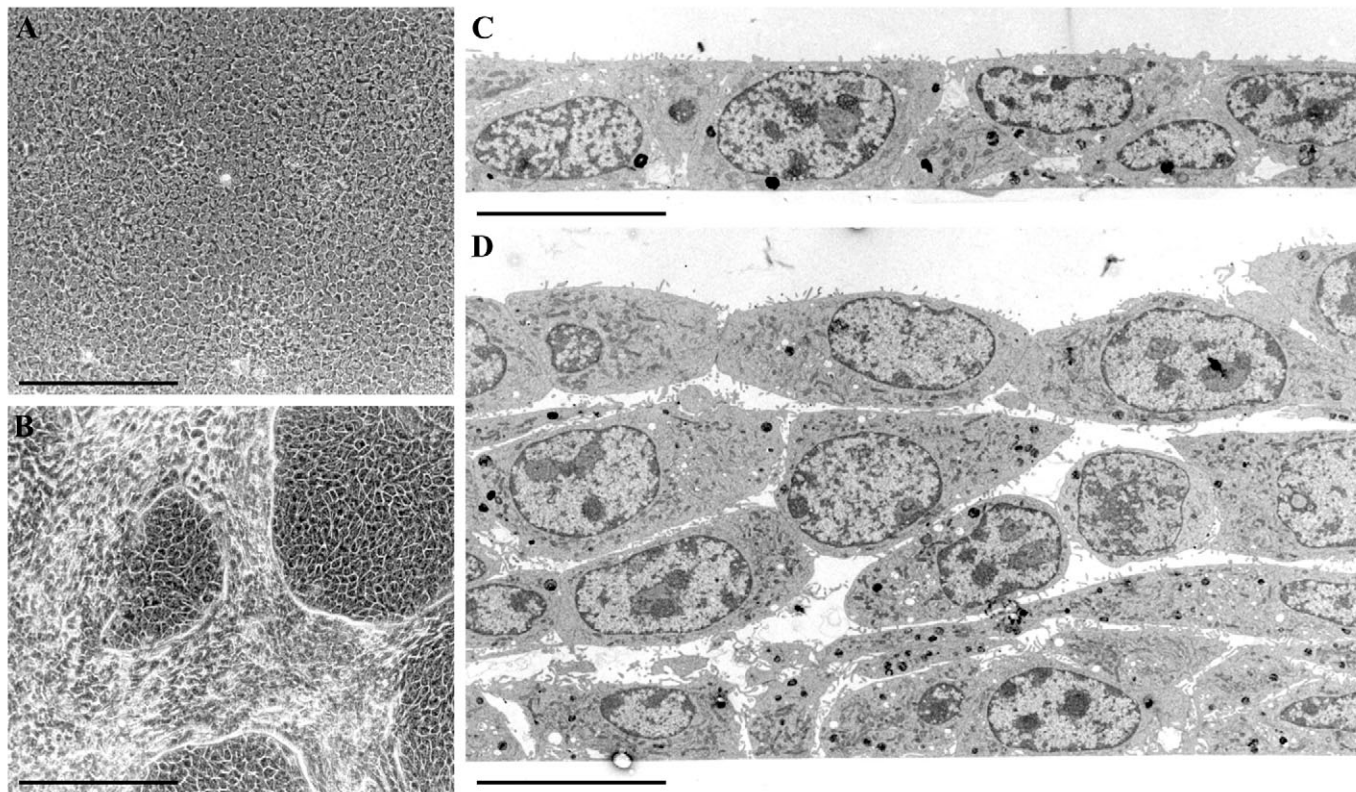
Fig. 5. TNF-α disrupts contact-mediated inhibition of cell proliferation. 2A4 cells were seeded at saturation density within a glass cylinder placed in the centre of a 22 mm well. After 24 hours, the cylinder was removed and the monolayer was incubated with or without TNF-α. (A) When left untreated for 9 days, the initial cobblestone-like monolayer of 2A4 cells remain strictly contact inhibited. (B) Over the same time period, 2A4 cells treated with 5 ng ml^{-1} TNF-α have formed thick ridges overlying the initial monolayer. (C) Thin section perpendicular to the plane of a control culture showing that the cells grow as a single layer. (D) Thin section perpendicular to the plane of a culture treated with 3 ng ml^{-1} TNF-α. The cells pile up forming multiple cell layers. Scale bars, 200 μm (A,B); 10 μm (C,D).

constitutively expressed by 2A4 cells but not ostensibly modulated by TNF-α (data not shown). Altogether, the results described above suggest that MMP-9-mediated pericellular matrix remodelling plays a role in TNF-α-induced 3D scattering.


Ability of TNF-α to promote 3D scattering is dependent on α2β1 integrin

The finding that TNF-α induces 2A4 cells to scatter and migrate within a 3D collagen matrix suggested that this cytokine might modulate cell interactions with type-I collagen. To test this hypothesis, we analysed the potential effect of TNF-α on the adhesion of 2A4 cells to collagen-coated dishes. As shown in Fig. 8A-C, pretreatment of 2A4 cells with TNF-α resulted in a greater than twofold increase in their attachment to native type-I collagen, whereas adhesion to gelatine (i.e. heat-denatured collagen) was not significantly modified.
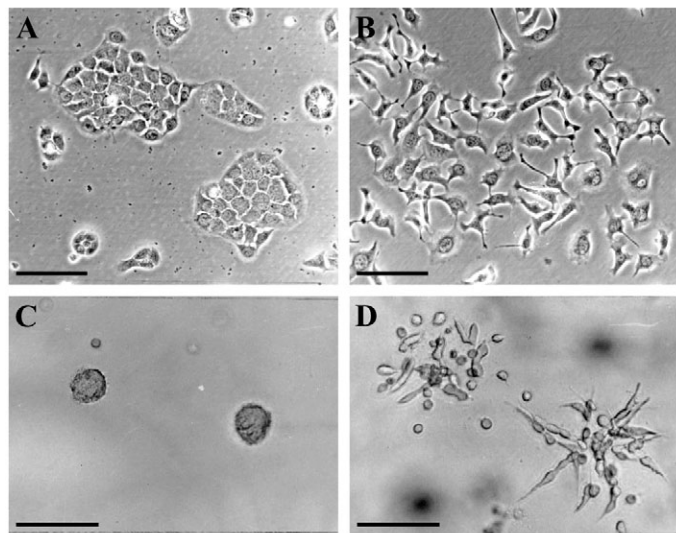
Fig. 6. TNF-α-induced cell scattering is mediated by TNF-R1. (A,B) 2A4 cells were seeded in plastic wells in DMEM-F12 medium supplemented with ITS+ Premix and grown for 24 hours. The cultures were then incubated for 2 hours with 40 μg ml^{-1} hamster monoclonal antibodies to either mouse TNF-R1 or TNF-R2 before addition of 10 ng ml^{-1} TNF-α. Pictures were taken 72 hours later. TNF-α-induced scattering is blocked by the anti-TNF-R1 antibody (A) but not by the anti-TNF-R2 antibody (B). (C,D) 2A4 cells were suspended in a collagen gel in defined medium. Goat irrelevant IgG (C) or goat anti-TNF-R1 agonist antibody (50 μg ml^{-1}) (D) were added both to the gel at the time of cell embedding and to the medium above the gel. The anti-TNF-R1 agonist antibody has induced 3D scattering of 2A4 cells (D). Scale bars, 10 μm.
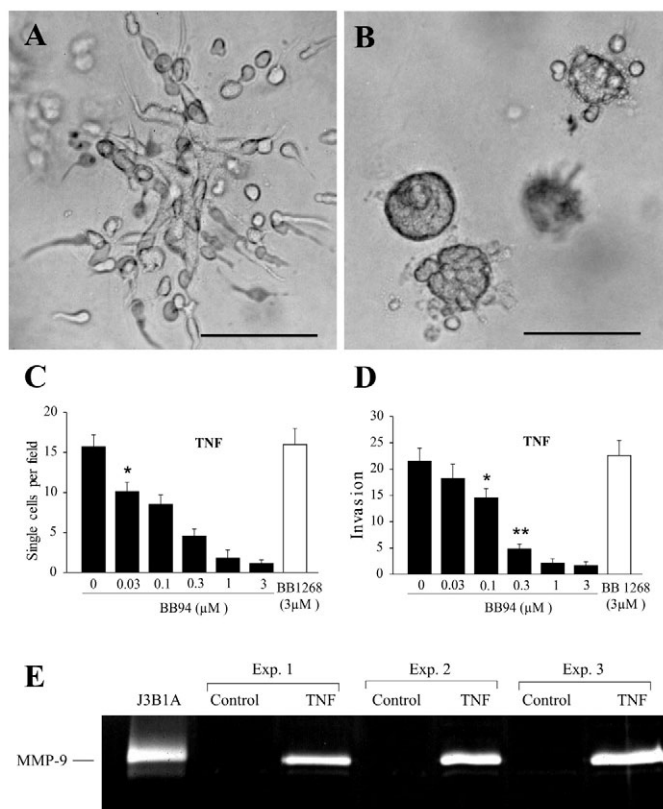
Fig. 7. TNF-α-induced 3D scattering and invasion of collagen gels require metalloproteinase activity. (A,B) 2A4 cells were grown in collagen gels in defined medium for 3 days and subsequently incubated for an additional 6 days with 10 ng ml⁻¹ TNF-α together with either the synthetic metalloproteinase inhibitor BB94 (1 μM) (B) or the inactive isomer BB1268 (3 μM) (A). Scale bars, 100 μm. (C) Quantification of the effect of increasing concentrations of BB94. 3D scattering is inhibited in a dose-dependent manner by BB94 (black columns) but is not affected by the inactive isomer BB1268 (open column). Quantification of 3D scattering was carried out after 6 days of treatment. Data represent the mean number of single cells per photographic field ± s.e.m. from at least three independent experiments. $*P<0.01$ compared with cultures incubated with TNF-α alone. (D) Inhibition of collagen-gel invasion by BB94. 2A4 cells were plated onto a collagen gel and subsequently incubated with 10 ng ml⁻¹ TNF-α in the presence of either BB94 or the inactive isomer BB1268. 5 days later, invasion was quantified by counting the number of all cellular structures (i.e. apparently single cells and cell cords) present in a photographic field at a focal plane beneath the surface monolayer. $*P<0.05$ compared with cultures incubated with TNF-α alone; $**P<0.0005$ compared with cultures incubated with TNF-α alone. (E) By gelatine zymography, proteolytic activity corresponding to the molecular weight of MMP-9 is not detectable in conditioned media from untreated 2A4 cells but is clearly present in conditioned media from TNF-α-treated cells. Conditioned media from murine J3B1A cells were used as a migration standard for MMP-9. The results illustrated were obtained with conditioned media collected from three independent experiments.

Cell-matrix interactions are largely mediated by integrins, a family of heterodimeric transmembrane receptors composed of non-covalently associated α and β subunits. Given that α2β1 integrin is the primary cell-surface receptor for fibrillar type-I collagen (Jokinen et al., 2004), we examined the potential

effect of TNF-α on the expression of the α2 subunit by flow cytometry. We found that TNF-α induced an increase of ~3.8 times in the surface expression of the α2 subunit (Fig. 8D,E). Because the latter pairs exclusively with β1 subunits to form functional α2β1 heterodimers (Ivaska and Heino, 2000), these findings imply that TNF-α induces a robust increase in cell-surface expression of α2β1 integrin.

Based on the results described above, we next hypothesized that increased α2β1-integrin-mediated interaction with type-I collagen fibrils might be required for the TNF-α-induced migration of 2A4 cells into the surrounding matrix. If so, one would expect to observe inhibition of cell scattering upon blockade of α2β1-ligand interactions. To verify this hypothesis, the 3D scattering assay was performed in the presence of a function-perturbing antibody that blocks the β1-integrin subunit. Addition of increasing concentrations of function-blocking antibody to collagen-gel cultures caused a dose-dependent suppression of TNF-α-induced 3D scattering. Notably, when added at 10 μg ml⁻¹, the antibody fully restored the formation of compact colonies that is normally observed in the absence of TNF-α. By contrast, a control antibody (isotype-matched anti-TNP IgM) had no inhibitory effect on cell scattering (Fig. 8F-H). These data imply that functional β1 integrins are required for TNF-α-induced 3D scattering of 2A4 cells.

The β1 subunit can associate with different α subunits to form at least 11 different heterodimers (Ivaska and Heino, 2000). To assess whether TNF-α-induced 3D scattering was dependent on α2β1 integrin, we took advantage of rhodocetin, a snake-venom integrin inhibitor that selectively blocks the α2β1 heterodimer (Eble et al., 2001). Rhodocetin binds with high affinity to α2β1 integrin and selectively suppresses cell interactions with type-I collagen, thereby acting as a specific antagonist of α2β1-integrin-mediated cellular responses (Eble et al., 2002). We found that rhodocetin markedly inhibited the adhesion of untreated 2A4 cells to collagen-coated dishes. Rhodocetin also caused a significant 31.6% decrease in the number of cells adhering to collagen following TNF-α pretreatment, although adhesion was not reduced to the low levels observed with untreated cells (Table 1). These findings suggested that α2β1 integrin plays an important role in TNF-α-induced increase in cell-collagen adhesion but that additional integrin or non-integrin collagen receptors might be involved as well. Importantly, however, when added to 2A4 cells grown in collagen gels, rhodocetin almost completely abrogated the TNF-α-mediated 3D scattering (Fig. 8I,L), thereby demonstrating that α2β1 integrin has an essential role in the invasive response of 2A4 cells to TNF-α. Finally, we examined the potential effect of rhodocetin on TNF-α-induced 2D scattering, cell proliferation and multilayering in postconfluent cultures. Although rhodocetin had no inhibitory activity on 2D scattering and cell proliferation, it prevented TNF-α-induced cell multilayering (data not shown). Taken together, these findings indicate that the different biological effects elicited by TNF-α on 2A4 cells have distinct requirements for α2β1 integrin.

Discussion

Although TNF-α was originally identified and characterized by its ability to induce tumour necrosis (Carswell et al., 1975;
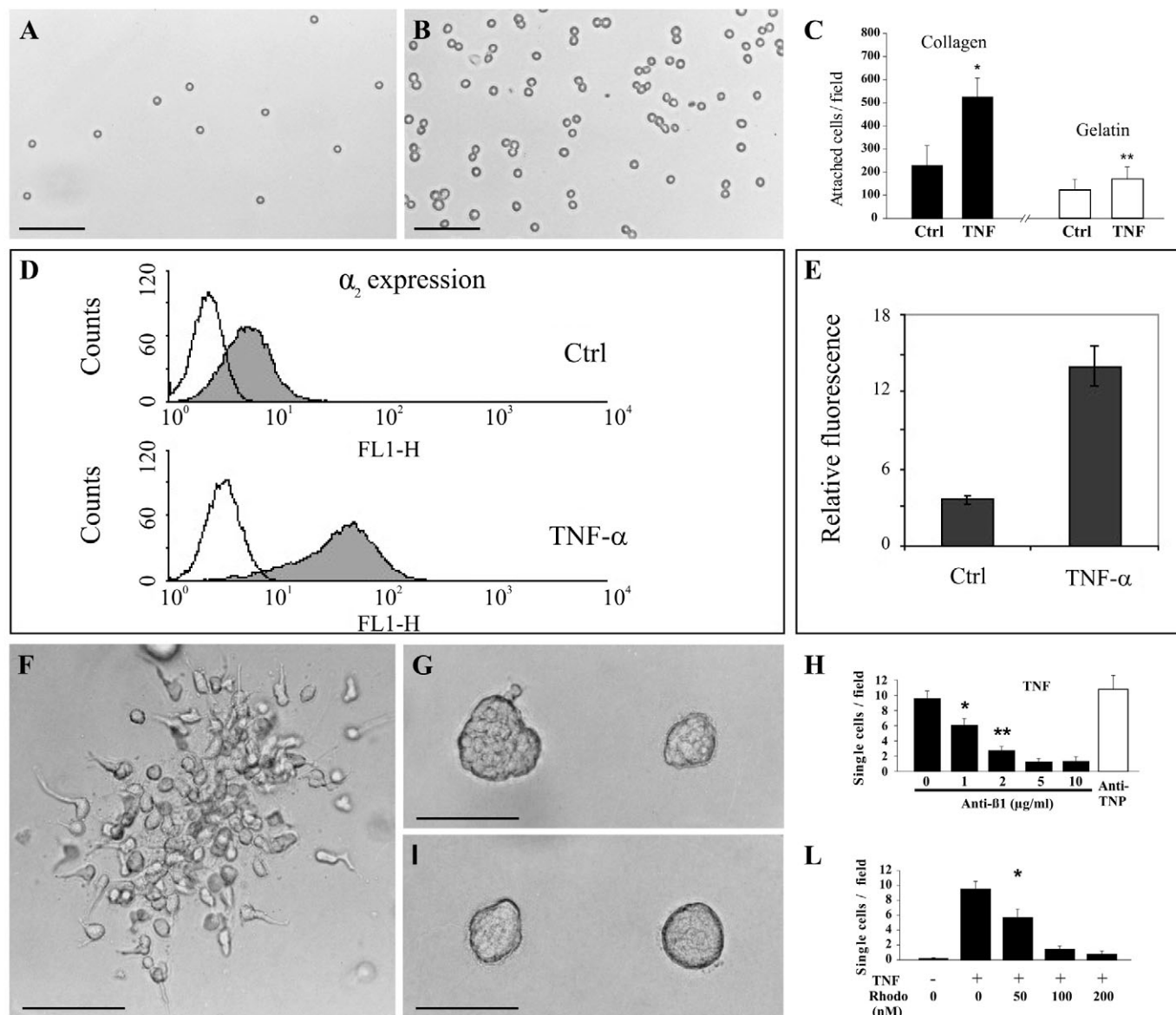
Fig. 8. See next page for legend.

Old, 1985), more recent studies indicate that this cytokine might act as an endogenous tumour promoter. Thus, overproduction of TNF-α increases the metastatic potential of tumour-cell lines (Malik et al., 1990) and treatment of mice with TNF-α promotes the development of liver metastases (Orosz et al., 1993). Moreover, mice deficient in TNF-α are resistant to skin carcinogenesis (Moore et al., 1999) and antibodies to TNF-α inhibit the development of experimental skin tumours (Scott et al., 2003). In an attempt to reconcile the apparently paradoxical activities of TNF-α, it has been proposed that the effects of TNF-α on tumour development are context dependent. Thus, high-dose local delivery of TNF-α can cause tumour regression, yet sustained production of endogenous TNF-α in the tumour microenvironment might actually enhance cancer development and spread (Balkwill, 2002).

Despite increasing evidence supporting a role for TNF-α in

tumour progression, the underlying molecular mechanisms are still poorly understood (Arnott et al., 2002). We have used the 2A4 mammary-epithelial-cell line to assess the effects of TNF-α on epithelial-cell adhesion and invasiveness. We found that TNF-α disrupts normal cell-cell adhesion and promotes a pronounced 3D scattering of preformed epithelial colonies, which culminates in the release of individual cells into the surrounding matrix. Notably, TNF-α also induces 3D scattering in J3B1A (Montesano and Soulie, 2002) mammary epithelial cells (our unpublished observation), which indicates that the phenomena described here are not unique to the 2A4 cell line.

It has previously been shown that TNF-α stimulates 2D scattering and motility in several epithelial tumour-cell lines (Rosen et al., 1991), as well as in untransformed human mammary epithelial cells (Chen et al., 2004). It was not known, however, whether TNF-α could induce cell dissociation and

Fig. 8. TNF-α-induced 3D scattering is dependent on α2β1 integrin. (A-C) TNF-α increases the adhesion of 2A4 cells to collagen-coated dishes. 2A4 cells were harvested from confluent cultures following a 48-hour preincubation in the absence (A) or presence (B) of 10 ng ml^{-1} TNF-α in defined medium and were seeded into collagen- or gelatine-coated bacteriological dishes. After a 60 minute incubation at 37°C, nonadhering cells were washed away and adhered cells were fixed and photographed using an inverted photomicroscope. Nonspecific adhesion to dishes that had not been coated with collagen was negligible. Scale bars, 100 μm. (C) Quantification of cell adhesion. Five randomly selected fields were photographed in each dish and the number of attached cells per field was counted on positive prints. Results represent the mean number of cells per field ± s.e.m. from three independent experiments. Mean values were compared using Student's unpaired *t* test. *$P<0.001$ versus control values; **not significantly different from control values. (D,E) TNF-α increases cell surface expression of the α2 integrin subunit. Cells were trypsinized and stained with monoclonal antibody against the mouse α2 subunit followed by secondary FITC-conjugated antibody, and were analysed by flow cytometry. (D) The plots show 2A4 cells incubated with monoclonal antibody against the α2 integrin subunit (grey profile) or secondary antibody alone (open profile) after 48 hours without (top) or with (bottom) treatment with 10 ng ml^{-1} TNF-α. The flow-cytometric plots shown are representative of five independent experiments. (E) Cells were treated with TNF-α and incubated with monoclonal antibody against α2-integrin subunit as described in D. Mean fluorescence intensities ± s.e.m. from five independent experiments were normalized to secondary antibody fluorescence. Mean values were compared using Student's unpaired *t* test. $P<0.005$ for values of TNF-α versus control. (F-H) Blocking antibodies to β1 integrins abrogate TNF-α-induced 3D scattering in a concentration-dependent manner. Cells were grown in collagen gels in defined medium for 3 days and were subsequently incubated for an additional 6 days with 10 ng ml^{-1} TNF-α in the absence (F) or presence (G) of function-blocking antibody against β1 integrin (10 μg ml^{-1}). (H) Dose-response analysis of the effect of increasing concentrations of anti-β1-integrin antibody. An isotype-matched irrelevant (anti-TNP) antibody was used as a control (open column). Quantification of 3D scattering was carried out after 6 days of treatment. Data represent the mean number of single cells per photographic field ± s.e.m. from at least three independent experiments. *$P<0.025$ compared with cultures incubated with TNF-α alone; **$P<0.0005$ compared with cultures incubated with TNF-α alone. (I,L) The α2β1-integrin antagonist rhodocetin abolishes TNF-α-induced 3D scattering. (I) Cells were grown in collagen gels in defined medium for 3 days and subsequently incubated for an additional 6 days with 10 ng ml^{-1} TNF-α in the presence of 200 nM rhodocetin. (L) Dose-response analysis of the effect of increasing concentrations of rhodocetin. *$P<0.0125$ for 50 nM rhodocetin compared with cultures incubated with TNF-α alone. (F,G,I) Scale bars, 100 μm.

Table 1. Effect of rhodocetin on cell adhesion to collagen

	Number of attached cells per field	
	Control	Rhodocetin
Untreated	159.86±33.57	61±17.13
TNF-α	371.93±19.44	254.46±12.02*

2A4 cells harvested from confluent cultures following a 48 hour preincubation with or without 10 ng ml^{-1} TNF-α were seeded into collagen-coated dishes in the presence or absence of 200 nM rhodocetin. Data represent the mean number of cells per field ± s.e.m. from three independent experiments and are compared using Student's unpaired *t* test.
*$P<0.001$ versus TNF-α values.

signalling. The difference in TGF-β requirement in the present study and that of Bates and Mercurio (Bates and Mercurio, 2003) might arise from the distinct origins (mammary vs intestinal) of the cell lines used.

In addition to inducing cell dispersion in 3D collagen gels, TNF-α also stimulated cell scattering in conventional cultures. Although 2D scattering approximates the in vivo complexity less closely than does migration within a 3D matrix, it provides a faster assay that is more amenable to pharmacological manipulation. Thus, 2D scattering provides a convenient readout in studies aimed at dissecting the intracellular signalling pathways that mediate TNF-α-induced cell motility (our unpublished observation).

Cell scattering is a manifestation of epithelial plasticity and is often part of a complex genetic programme resulting in an epithelium-mesenchyme transition (EMT) (Grunert et al., 2003). However, several observations suggest that the TNF-α-induced changes reported in this study do not reflect a bona fide EMT. First, 2D scattering was rapidly reversible upon removal of the cytokine (Fig. 2), even after the cells were continuously exposed to TNF-α for 3 weeks (our unpublished observation). Second, TNF-α neither downregulated the production of epithelial proteins (e.g. cytokeratins) nor induced the production of mesenchymal proteins (e.g. vimentin) in 2A4 cells. Third, TNF-α treatment was associated with only a modest decrease in the production of E-cadherin (our unpublished observation). We therefore consider that TNF-α-induced changes are more accurately described as cell scattering than EMT.

TNF-α exerts its biological effects through the activation of two distinct cell-surface receptors, TNF-R1 and TNF-R2. TNF-R1 is constitutively expressed and virtually ubiquitous, whereas TNF-R2 is regulated, has a more restricted distribution and mediates only a subset of TNF-α activities (Aggarwal, 2003; Baud and Karin, 2001; Dempsey et al., 2003; Locksley et al., 2001; MacEwan, 2002; Varfolomeev and Ashkenazi, 2004). Our results indicate that the effects of TNF-α on cell dispersion are mediated through the activation of TNF-R1, as shown by the suppression of cell scattering by antibodies that block TNF-R1 but not by antibodies that block TNF-R2, and the induction of cell scattering by an agonist anti-TNF-R1 antibody.

Cell migration through the ECM relies on extracellular proteolysis and is largely mediated by MMPs, a family of zinc-dependent endopeptidases that, collectively, are able to degrade virtually all proteins of the ECM and also cleave several surface-associated proteins (Egeblad and Werb, 2002). In agreement with previous studies in mammary epithelial cells

migration in a 3D ECM environment. While this study was in progress, Bates and Mercurio (Bates and Mercurio, 2003) reported that TNF-α cooperates with TGF-β to induce the disintegration of colon carcinoma organoids grown in collagen gels but is unable on its own (i.e. in the absence of exogenous TGF-β) to induce cell scattering. In light of these findings, we used a blocking antibody to TGF-β1 as well as a selective inhibitor of the TGF-β type-I receptor to ascertain whether an autocrine TGF-β loop could be involved in TNF-α-induced 3D scattering of 2A4 cells. Neither reagent inhibited 3D scattering of 2A4 cells (our unpublished data), suggesting that, in our system, the effect of TNF-α is not dependent on TGF-β

(Lee et al., 2000; Chen et al., 2004), we found that TNF-α induces the production of MMP-9 (92 kDa gelatinase) in 2A4 cells. Notably, gelatinases are thought to play an important role in collagen degradation by acting sequentially after the initial cleavage of the triple helix by constitutively produced MMPs such as MT1-MMP or MMP-1 (Creemers et al., 1998). The functional relevance of MMP in our system is supported by the finding that TNF-α-induced 3D scattering is almost totally suppressed by the synthetic MMP inhibitor BB94 but not by the related inactive isomer BB1268. A straightforward explanation for MMP requirement in TNF-α-induced 3D scattering is that, owing to their collagen-degrading ability, MMPs release the mechanical restraints imposed on the cells by the surrounding matrix, thereby allowing them to migrate throughout the collagen gel. It is possible, however, that MMPs promote cell invasiveness by more complex mechanisms, such as by cleaving E-cadherin, by processing cell-surface receptors for matrix components, by exposing cryptic sites in ECM proteins or by releasing proteolytic fragments of ECM proteins that stimulate cell motility (Egeblad and Werb, 2002). Members of the ADAM family of metalloproteinases, which are also inhibited by BB94, have been implicated in TNF-α-induced ectodomain shedding of membrane-bound EGF-like ligands, resulting in EGF receptor (EGFR) transactivation (Argast et al., 2004; Chen et al., 2004). However, autocrine signalling through the EGFR is unlikely to mediate 3D scattering of 2A4 cells in collagen gels, because this phenomenon is neither prevented by an EGFR tyrosine-kinase inhibitor nor induced by EGF-family ligands, including EGF itself, TGF-α, heparin-binding EGF-like growth factor (HB-EGF), β-cellulin and amphiregulin (our unpublished observation).

In addition to requiring metalloproteinase activity, TNF-α-induced 3D scattering appears to involve the modulation of cell-matrix interactions. Thus, we found that TNF-α induces a twofold increase in cell adhesion to type-I collagen as well as an increase of ~3.8 times in the surface expression of the α2 subunit of α2β1 integrin, which is the primary cell-surface receptor for fibrillar type-I collagen (Jokinen et al., 2004). Because the α2 chain exclusively associates with a β1 chain to form an α2β1 heterodimer, these findings imply that TNF-α induces a robust increase in the cell surface expression of α2β1 integrin, a conclusion that is consistent with previous studies in other cell types (Kawashima et al., 2001; Nista et al., 1996). Importantly, the functional relevance of α2β1 integrin in our experimental model is supported by the demonstration that TNF-α-induced 3D scattering is suppressed by addition of either a β1-integrin-blocking antibody or the specific α2β1-integrin antagonist rhodocetin. Previous work has generated conflicting data concerning the involvement of α2β1 integrin in cell migration. Whereas some studies suggest that α2β1 integrin promotes cell migration (Lochter et al., 1999; Valles et al., 1996; Vihinen et al., 1996; Yamada et al., 1990), others indicate that expression or activation of α2β1 integrin decreases cell invasion of the surrounding collagen matrix (Baeckstrom et al., 2000; Zutter et al., 1995). Using phenotypically normal mammary epithelial cells, we have shown here that interfering with α2β1-integrin function abrogates 3D cell scattering. This inhibitory effect might be the consequence of disturbed cell adhesion to the collagen fibrils that form the 3D gel. However, it is also possible that blockade of α2β1 integrin disrupts cell interactions with endogenously produced ECM. In addition to acting as a collagen receptor, α2β1 integrin binds laminin-1 (Colognato et al., 1997) and laminin-5 (Decline and Rousselle, 2001), and might therefore mediate cell adhesion to endogenously deposited laminin molecules. Finally, TNF-α-induced cell scattering might involve α2β1-integrin-transduced intracellular signalling (Inoue et al., 2003). In this respect, it is of interest that α2β1 integrin has been shown to regulate the production of MMPs (Lochter et al., 1999).

We have provided evidence that, as well as promoting cell migration and matrix invasion, TNF-α confers on 2A4 cells several additional properties that are characteristic of malignantly transformed cells. Thus, TNF-α stimulates the proliferation of 2A4 cells under growth-factor deprivation. It also promotes anchorage-independent growth and disrupts normal contact-mediated inhibition of cell proliferation, resulting in focal multilayering. These findings confirm and extend the results of previous reports showing that TNF-α exerts mitogenic activity (Argast et al., 2004; Chen et al., 2004; Iocca and Isom, 2003; Ip et al., 1992; Kaiser and Polk, 1997) and induces anchorage-independent growth (Okamoto and Oyasu, 1997) in some types of epithelial cells. Our studies significantly expand those earlier observations by demonstrating that TNF-α not only acts as a potent mitogen for mammary epithelial cells but also subverts the growth-suppressive mechanisms that normally operate in postconfluent monolayer cultures. The mechanisms responsible for the growth-promoting activity of TNF-α are not known but preliminary results suggest that, unlike 3D scattering, TNF-α-induced cell proliferation of 2A4 cells involves the generation of a metalloproteinase-dependent autocrine loop leading to EGFR transactivation (our unpublished observation).

TNF-α has been proposed to play a role in normal mammary gland development, owing to its ability to stimulate branching morphogenesis (Ip et al., 1992; Varela and Ip, 1996). Intriguingly, α2β1 integrin has also been implicated in mammary branching morphogenesis (Chen et al., 2002). In apparent contradiction to those studies, we report here that TNF-α induces the disaggregation of epithelial colonies and promotes invasion of a 3D collagen matrix. How can these seemingly paradoxical findings be reconciled? It is relevant in this respect to consider that induction of cell migration and remodelling of cell-matrix interactions can impinge on both normal organogenesis and the acquisition of invasive potential during tumour progression. Thus, in the context of a developing epithelium endowed with normal cell-cell adhesion properties, activation of cell motility and/or modulation of integrin expression are believed to promote coordinated cell rearrangements, culminating in tissue morphogenesis. By contrast, in the context of a premalignant epithelium with dysfunctional cell-cell interactions, the same cellular responses could result in the disruption of normal tissue organization and induction of cell invasiveness. In light of these notions, we propose that TNF-α has a context-dependent dual activity on mammary epithelial cells. When produced in a spatially and temporally restricted manner in the developing mammary gland, TNF-α might promote branching tubulogenesis (Ip et al., 1992; Varela and Ip, 1996). However, when chronically produced at sustained high levels in a precancerous tissue, TNF-α might endow epithelial cells with invasive properties

and subvert the physiological mechanisms that restrain cell proliferation, thereby promoting tumour progression. A similar duality of function has previously been attributed to HGF, a motility-inducing factor that, depending on the environmental context, promotes either branching morphogenesis (Montesano et al., 1991a) or cell invasiveness (Trusolino and Comoglio, 2002; Zhang and Vande Woude, 2003).

In conclusion, our demonstration that TNF-α causes disaggregation of cohesive colonies of mammary epithelial cells and invasion of the surrounding collagen matrix provides a mechanistic basis for the reported ability of this cytokine to promote tumour progression and cancer-cell dissemination (Szlosarek and Balkwill, 2003). In addition, our findings that TNF-α strongly upregulates cell-surface expression of α2β1 and that this integrin is essential for TNF-α-induced invasiveness suggest that modifications of α2β1 levels in tumours could be used as an indicator of the therapeutic efficacy of TNF-α antagonists (Palladino et al., 2003).

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