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TNFα increases *in vitro* migration of human HPV18-positive SW756 cervical carcinoma cells

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Key words: TNFα, migration, carcinoma, SW756, wound healing assay.

ABSTRACT: TNFα has been associated with both, tumor survival and apoptosis. This cytokine is also involved in promoting cell migration during wound healing and tumorigenesis. SW756 is a HPV18-positive cervical carcinoma cell line, which has been used to study different mechanisms of cervical cancer progression. An *in vitro* assay of scratch wound healing onto monolayers of SW756 cells was used to assess the effect of TNFα on cell migration into a wound space. It was found that SW756 cells have the ability to migrate, but not proliferate in response to scratch wounding in a serum-free medium supplemented with TNFα. RT-PCR analysis showed that SW756 cells express TNFα mRNA when incubated in medium with and without serum. Wound closure and migration rate of SW756 cells were significantly increased in the presence of serum-free media supplemented with TNFα (10 ng/mL) as compared to serum-free media, and media supplemented with either anti-TNFα antibody or both TNFα and anti-TNFα antibody ($p<0.05$). The results showed a stimulatory effect of TNFα on the migration of SW756 cervical carcinoma cells, suggesting a novel and important role for TNFα in cervical cancer progression.

Introduction

Tumor cell invasion of connective tissue and blood vessels is one of the key steps in the metastatic spread of cancer. Evidence for the role of TNFα in increased cell migration has been demonstrated *in vitro* in cultured keratinocytes, and *in vivo* in murine tumor promotion models (Scott *et al*., 2004). TNFα has also been identified as a chemotactic factor for inflammatory cells involved in metastasis, such as mast cells (Olsson *et al*., 2003). In cervical cancer, little is known of the role of TNFα in migration of transformed cells.

Epidemiological studies have demonstrated a causal relationship between chronic infection of the uterine cervix by human papillomavirus (HPV) and cervical neoplasia (De Boer *et al*., 2004). HPVs are a family of DNA viruses with over 150 genotypes. More than 40 of these genotypes infect the anogenital tract, causing a variety of abnormalities ranging from genital warts to invasive cancer. Certain types are considered more carcinogenic in humans, such as HPV16 and HPV18, which are associated with adenocarcinomas and squamous cell carcinomas of the cervix, respectively (Woodman *et al*., 2003). Recent genetic studies support the hypothesis that constitutionally determined TNFα polymorphisms might influence response to high risk HPV infection, and therefore, susceptibility to cervical neoplasia (Kirkpatrick *et al*., 2004).

SW756 is the code for an HPV18-positive cervical carcinoma cell line, which is used to study the different mechanisms of cervical cancer progression. It has been shown that SW756 cells do not synthesize β-interferon, an antiangiogenic and antimitotic cytokine (Berger and Hawley, 1997), in response to TNFα stimulation

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(Bachmann *et al*., 2002). However, this cell line is believed it might maintain its ability to synthesize TNFα through NF-κB activation (Altenburg *et al*., 1999), probably through $TNFR_1$ (Park *et al*., 2003) or CD40R, both members of the TNF receptor family whose activation induces NF-κB translocation (Berverich *et al*., 1994). The SW756 cell line also produces high levels of IL-6, which in turn stimulates monocyte chemoattractant protein-1 (MCP-1) production, however, due to the lack of sgp80, a key protein in the IL-6 signaling pathway, they are unresponsive to IL-6 stimulation (Hess *et al*., 2000; Smola-Hess *et al*., 2001).

In vitro wound healing assays have been used with multiple cell types and are a classic and commonly used method for studying cell migration and the biology underlying it. In this study we developed an *in vitro* assay of scratch wound healing to assess if TNFα had a significant effect on SW756 migration.

Materials and Methods

Cells, reagents and antibodies

The human cervical carcinoma SW 756 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). Reagents were purchased from Sigma (St. Louis, MO, USA). Recombinant human soluble TNFα was purchased from Boehringer Manheim Biochemica (Manheim, Germany). Anti-TNFα Ab neutralizing rhTNFα (Clone B-C7) was purchased from Biosource International (Camarillo, CA, USA). Polyclonal rabbit antihuman Ki67 antibody and peroxidase-conjugated goat antirabbit IgG secondary antibody were obtained from Dako (Carpinteria, CA, USA). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TNFa were synthesized by Bios Chile (Santiago, Chile). Oligo-dT primer, dNTP mix, ribonuclease inhibitor RNasin and 15 U of AMV reverse transcriptase (AMV-RT) were purchased from Promega (Madison, WI, USA). Taq DNA polymerase, trizol and all culture media were was purchased from Gibco BRL (Gaithersburg, MD, USA). 3-3'diaminobenzidine (DAB) was obtained from Chemicon International, Inc (Temecula, CA, USA).

Wound healing assay

Culture conditions were optimized to ensure that cell monolayers were homogeneous maintaining the cell viability prior to wounding. In order to reduce cell proliferation for the experiments, cell cultures were grown to confluency and deprived of serum, as previously described (Geimer and Bade, 1991; Hinz *et al*., 1999; Wang *et al*., 2002; Fumoto *et al*., 2003). Some incubation conditions were modified for optimization of the experimental model as described below. The objective was to obtain a reproducible measurement of the migration of the wound edge towards the wound space with minimum cell proliferation but without loss of cell viability over the experimental time period.

SW756 cells were grown at 37ºC under humidified atmosphere of 5% CO_2 and 95% O_2, in L-15 Leivobitz, supplemented with 10% fetal bovine serum and 100 U/mL Penicillin and 100 µg/mL Streptomycin according to ATCC indications. For migration analysis cells were detached by trypsinization, resuspended in the same medium and plated in triplicate at $3x10^5$ cells per well of a 24-well plate (area 7 cm^2). After cell became confluent cells were starved in a RPMI serum free medium for 18 hrs, then one artificial wound per well was scratched into the monolayers with a sterile plastic 10 µL micropipette tip to generate a uniform wound that was devoid of adherent cells. After wounding, tissue culture medium was removed, and cells were washed at least twice in PBS to eliminate detached cells. Experiments were performed by incubating the cells with RPMI only (control) or supplemented with a) a saturating concentration of 10 ng/mL rhTNFα (Bechtel *et al*., 1996), b) 10 µg/mL anti-TNFα Ab, or c) a combination of both, to evaluate the effect of TNFα on cell migration into the wound space. Wound closure was monitored by digital photographs taken in a phase-contrast microscope (Nikon Diaphot 300, Tokyo, Japan) across the wound at the moment of wounding and at 24 hrs post-wounding. Micrographs were studied by videomicroscopy and image analysis.

Videomicroscopy and image analysis

The system consisted of a light microscope with phase-contrast illumination (Nikon 300, Japan) fitted with a TV camera (CoolSNAP-Pro, Media Cybernetics, MD. USA) and interfaced with a host computer, image processing (Image-Pro Express software, Media Cybernetics, MD. USA), and recording systems. The light microscope images were entered into the camera through a 4x objective and transferred to a color monitor. Before measuring, distance calibration was defined using a slide with a micrometric scale for microscopy (Rechert, Austria). Distances between the edges were measured by using the image analysis software at 50 µm intervals all along the wound. A minimum of 120

readings were made on each well. Quantitative wound closure assays were performed in triplicate wells and each experiment was repeated at least three times. This system provided sufficient resolution to determine the magnitude of wound closure and cell morphology at the wound margins. The results of the three experiments were pooled for statistical analysis.

RNA isolation, reverse transcription and polymerase chain reaction (PCR)

Lysis of SW756 cells was performed with TRIzol reagent, and total RNA was extracted according to the manufacturer's protocol. A solution containing 20 µg of total RNA, oligo-dT primer, dNTP mix, ribonuclease inhibitor RNasin, and 15 U of AMV reverse transcriptase (AMV-RT) in reaction buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl; 50 mM $MgCl_2$; 50 mM DTT; 2.5 mM spermidine) was incubated at 42°C for 60 min. The solution was heated to 90°C for 5 min to inactivate AMV-RT and then cooled at 4°C.

The following primer sets described by Baram *et al*. (2001) were synthesized: GAPDH sense 5′-CGGAGTCAACGGATTTGGTCGTAT, GAPDH antisense 5′-AGCCTTCTCCATGGTTGGTGAAGAC; and TNF-α sense 5′-CTGTACCTCATCTACTCC CAGGTC, TNF-α antisense 5′-AGACTCGGCAAAGTCGAGATAGT. The PCR was performed in a 25 µl reaction mixture (10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1-1.4 µM of 5' and 3' primers of each gene, and 1 unit of Taq DNA polymerase. cDNA was amplified for 35 cycles (Amplitron II, Barnstead/Thermolyne Corporation, Dubuque, IA, USA). Each cycle consisted of denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and primer extension at 72°C for 1 min. Before amplification, reactions were incubated at 94°C for 3 min. After amplification, extension was performed at 72°C for 10 min. Samples were electrophoresed, visualized in an ultraviolet transilluminator, photographed, and analyzed with NIH Image 1.61 (Bethesda, MD, USA).

Cell proliferation analysis

Cells were plated in 24-well plates in triplicate at a density of $3x10^5$ cells per well in the tissue medium described above for SW756. Then they were incubated in the serum free mediun with RPMI for 18 hrs and treated with TNFα (10 ng/mL). Cell counts were performed in duplicate at 24 hrs using a hemacytometer.

SW756 cells were grown on glass coverslips in a 12-well plate. After the wound was made and the cells were treated with TNFα (10 ng/mL) for 24 hrs, cells were washed with 10 mM Tris-HCl, pH 7.8 (TBS), fixed with paraformaldehyde for 30 min and permeabilized with TBS containing 0.1% Triton X-100 for 10 min. Subsequently, the permeabilized cells were treated with 10% H_2O_2 for 25 min and blocked in TBS/Tween 20 supplemented with bovine serum albumin for 1 hr and then incubated with (1:200) polyclonal rabbit anti-human Ki67 Ab for 24 hrs at room temperature and humidified atmosphere. Cells were then washed and incubated with (1:100) peroxidase-conjugated goat anti-rabbit IgG secondary Ab for 1 hr at room temperature. The reaction was developed with 3-3′-diaminobenzidine (DAB) and 3 mL/mL H_2O_2 in 50mM Tris (pH=7.6).

Trypan blue assay

For evaluation of cell monolayer damage produced by the 10 µL micropipette tip scratch wound and culture cell viability, SW756 cells were treated with 0.4% Trypan blue and immediately observed in a phase-contrast microscopy (Nikon Diaphot 300, Tokyo, Japan) all across the wound area to determine trypan blue exclusion (Yasui *et al*., 2003).

Statistical analysis

All data were tabulated and statistical tests were performed with JMP-IN 4.0.4 SAS Institute Inc. (Cary, NC, USA). All data are represented as the mean ± SEM of three independent experiments performed in triplicate. Significant statistical differences between groups were examined using unpaired ′t′ test and one way ANOVA and Tukey Kramer tests. The non-parametric Wilcoxon and Kruskal-Wallis tests were used when variables did not have a normal distribution. Differences were considered statistically significant when $p<0.05$.

Results

Effects of incubation with serum-free media on SW756 cell culture

Human SW756 cervical carcinoma cells positive for HPV-18 grew as monolayers, adherent to the culture flasks. Their proliferation rate was dependant on the initial cell concentration in each well. Optimum confluent monolayer cultures were obtained with an initial concentration of $3x10^5$ SW756 cells/mL in 24-

well flasks after 72 hrs of culture. Cells were incubated with L-15 Leivobitz culture media supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL Streptomycin, at 37ºC and 5% CO_2.

Replacement of culture media by a serum-free media resulted in proliferation inhibition with no alteration of cell viability, as confirmed by hemacytometer counting and trypan blue exclusion at 42 hrs of culture in serum-free media. The lapse of serum free media of 42 hrs corresponds to the 18 hrs before wounding and 24 hrs post-wounding, according to the following experiments. In fact, viability was maintained near 97% and the number of cell counts was similar at 0 and at 42 hrs after incubation with serum-free media. In addition, loss of the proliferation marker Ki67 expression was observed in confluent cells incubated with serum-free media and in confluent cells incubated with serum-free media supplemented with 10 ng/mL TNFα, as compared to SW756 cells growing in full media of L-15 Leivobitz, supplemented with 10% fetal bovine serum (Fig. 1).

SW756 cells express endogenous mRNA TNFα

To determine if SW756 express endogenous mRNA TNFα, and if this endogenous expression was affected by incubation with serum-free media, total RNA was extracted from SW756 cells incubated with serum supplemented media, and with serum-free media for 42 hrs, and subjected to RT-PCR to determine TNFα mRNA expression. The results show TNFα expression in SW756 cells after 42 hrs of serum starvation (Fig. 2).

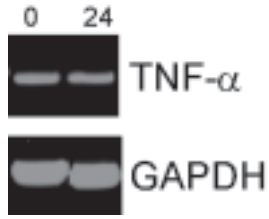

FIGURE 2. SW756 cell expression of TNFa mRNA is independent of serum presence in the culture medium. SW756 cells were grown to confluency in L-15 Leivobitz medium, supplemented with 10% fetal bovine serum (0h) as described in Methods, incubated with a serum free medium for 42 hrs (24h). Total mRNA was isolated, and TNFα mRNA expression was detected by RT-PCR.

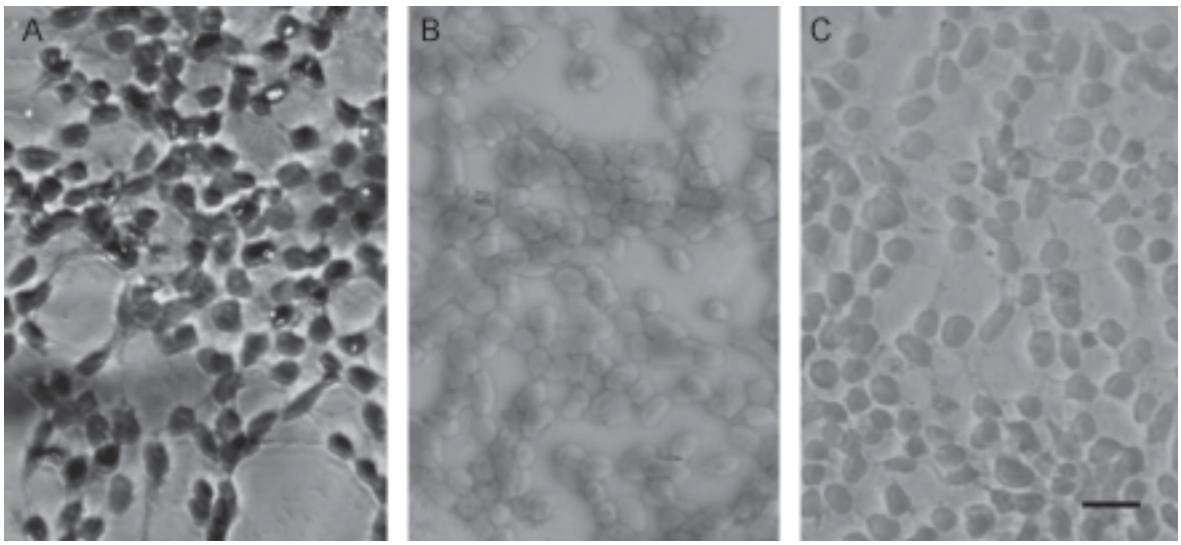

FIGURE 1. TNFα (10 ng/mL) does not promote expression of the proliferation marker Ki67 in SW756 cells incubated with serum-free medium. SW756 cells were grown on glass coverslips and Ki67 expression was detected by immunohistochemistry as described in Methods. Ki-67 protein is stained in black. A: Positive control: SW756 cells grown in L-15 Leivobitz medium, supplemented with 10% fetal bovine serum as described in methods. B: SW756 cells grown into confluence, incubated with a serum free medium for 18 hrs and then supplemented with 10 ng/mL TNFα for 24 h. C: Negative control: obtained by omitting the first Ab. Scale bar: 10 µm.

SW756 culture wounding

The size of the scratch wound created with a 10 µL micropipette tip ranged from 306 to 583 µm between wound edges. Damaged and non-viable cells were eliminated by PBS washing. Cell death as a result of scratch wounding was minimal and had no impact on cell viability at the wound edges as demonstrated by trypan blue exclusion. After 24 hrs of wounding a large amount of SW756 cells had already migrated into the wound space. Migrating cells exhibited an elongated morphology, with the longitudinal axis polarized toward the wound, which is a feature of well-organized migration. In addition, migrating cells presented similar size and morphology to growing cells before becoming confluent. In all the experiments the wound margin was observed rather diffuse due to active cell migration. At the wound edges uniform cell migration was observed. The same pattern was observed when TNFα was added to the medium (Fig. 3).

Effects of TNFα on wound closure and SW756 migration rate

SW756 cells, cultured as confluent monolayers in 24-well plates were wounded as described above, and incubated with serum-free media alone (control media), or serum-free media supplemented with either 10 ng/mL of rhTNFα, 10 µg/mL of TNFα neutralizing antibody (anti-TNFα), or 10 ng/mL rhTNFα and 10 µg/

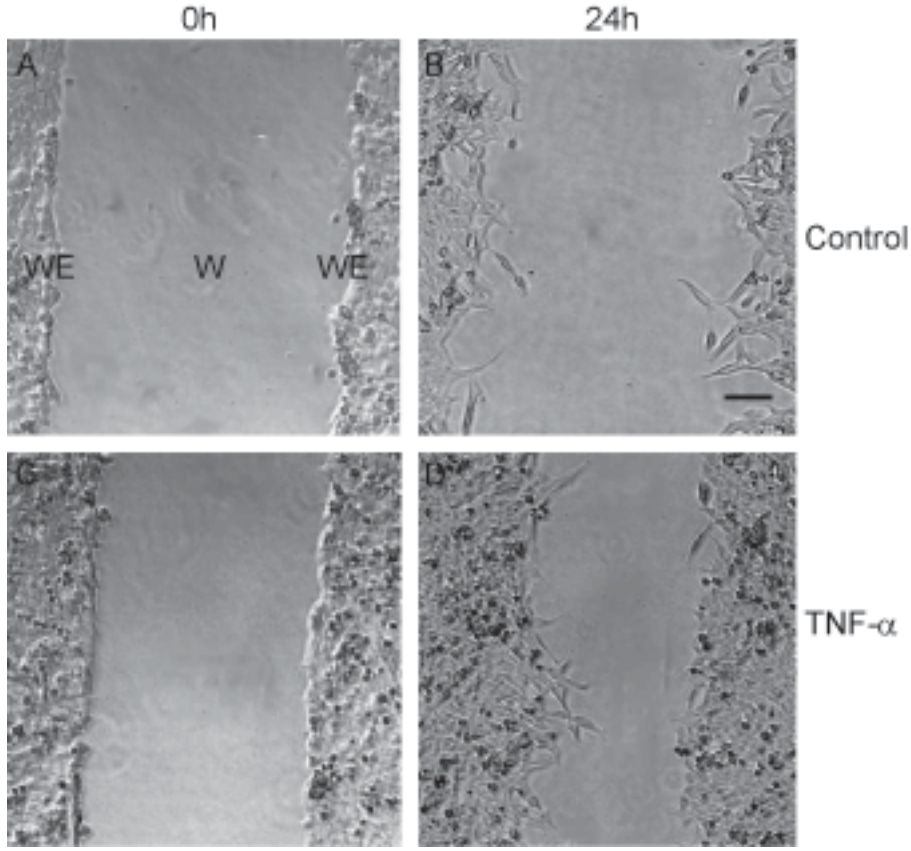

FIGURE 3. Effect of rhTNFα on SW756 cell migration. Representative phase-contrast images of cells migrating into the wounded area in an *in vitro* scratch wound healing assay. SW756 cells were grown into monolayers in triplicate in 24-well plates. Confluent cultures were starved for 18 hrs in RPMI, then scratched with a 10 mL pipette tip as described in the Methods section. Wound at time 0 (0h) for SW756 cells incubated with control, serum-free media (A) and with serum-free media supplemented with 10 ng/ml TNFα (C). (24h) 24hrs after wounding for SW756 cells incubated with control, serum-free media (B) and with control media supplemented with 10ng/ml TNFα (D). W= wound space, WE= Wound edge. Scale bar, 100 µm.

mL anti-TNF antibody (rhTNF-α/anti-TNFα).

First, changes in wound size (distance between wound edges) at 24 hrs post wounding were analyzed using the formula (wound size at 24 hrs/initial wound size) x 100. The results showed that addition of 10 ng/mL rhTNFα significantly increased wound closure as compared to all the other treatment conditions, control media, and control media supplemented with anti-TNFα and TNFα/anti-TNFα ($p<0.05$ ANOVA and Tukey Kramer tests). However, a significant increase in wound closure was also observed in the cells treated with media supplemented with rhTNF-α/anti-TNFα antibody, as compared to cells treated with control media or control media supplemented with anti-TNFα antibody ($p<0.05$) (Fig. 4).

In order to assess the effects of TNFα on SW756 cell migration, the rate of cell migration was determined at 24 hrs using the formula: ˚ (initial wound size - wound size at 24 hrs)/24. Between the time of wounding and 24 hrs after wounding, cells treated with 10 ng/mL rhTNFα supplemented media presented significantly higher migration rate as compared to cells treated with control media, control media with 10 μg/mL anti-TNFα, and control media with TNFα/anti-TNFα ($p<0.05$, Wilcoxon and Kruskal Wallis tests) (Fig. 5).

Discussion

Initially, TNFα was thought as a potentially effective anticancer therapeutic agent, due to its ability to kill some tumor cell lines *in vitro*, as well as, certain types of tumors *in vivo* (Haranata *et al*., 1984; Palladino *et al*., 1987; Creasey *et al*., 1986; Hagari *et al*., 1995). However, in the recent years new biological activities of TNFα, which could promote tumor growth and invasion, were identified. TNFα, a key mediator of inflammation (Locksley *et al*., 2001), acted as a mitogenic growth factor for human follicular dendritic cell lines (Park *et al.*, 2003), and in cervical fibroblasts of human uterus stimulated MMP-9 production, an enzyme with highly invasive activity and metastatic potential (Sato *et al*., 1996; Hofmann *et al*., 2000; Scott *et al*., 2004). In addition, TNFα acted on the endothelium stimulat-

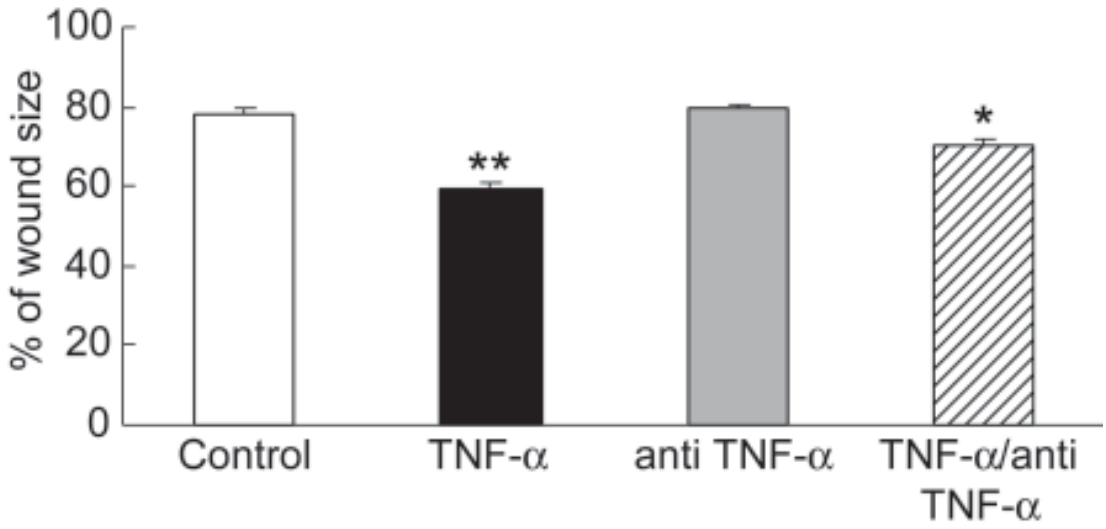

FIGURE 4. Effects of TNFα on wound closure of SW756 cells. Wound closure was monitored by digital photographs taken in a Nikon phase-contrast microscope across the wound at the moment of wounding and at 24 hrs after wounding. Wound closure was assessed as the distance between the wound edges, calculated and quantified using an Image Pro-Express software 4.01. The percentage of wound closure was evaluated using the formula (wound size at 24 hrs/ initial wound size) x 100. Data are shown as mean ± SEM from 3 independent experiments done in triplicate.
** $P<0.05$ increased wound closure for TNFα incubated cells as compared to control, antiTNFα, and antiTNF/TNFα (Anova and Tukey Kramer tests).
*$P<0.05$ increased wound closure for TNFα/antiTNFα as compared to control and antiTNFα, and reduced wound closure as compared to TNFα. (Anova and Tukey Kramer tests)

ing angiogenesis (Norrby, 1996; Secchiero *et al*., 2004) and leukocyte recruitment (Walsh *et al.,* 1991).

In vitro wound healing assays are useful for the study of the mechanisms involved in cell migration in multiple cell types (Lampugnani, 1999; Huang *et al*., 2003; Yarrow *et al*., 2004). Although it seems a simple assay, experimental conditions differ significantly depending on the type of cell line and the parameters to be assessed. The scratch wound healing assay used in these studies had the purpose of analyzing the role of TNFα on SW756 cell migration. It was found that SW756 carcinoma cell line, positive for HPV-18, in a serum-free environment, migrated in response to a scratch wound and was able to express endogenous TNFα mRNA. When exogenous TNFα was added to the serum-free media, this migration ability was significantly increased as measured at 24h after wounding. This TNFα effect on migration was partially, but significantly inhibited by the addition of an anti-TNFα antibody to the serum-free media. In addition, SW756 migration into the wound space, in the absence of exogenous TNFα, seemed to be independent of the endogenous TNFα produced by these cells, since addition of anti-TNFα to the serum free media did not affect cell migration. Therefore, other growth factors or cytokines produced in response to wounding could be involved in stimulating SW756 cell migration, which should be further analyzed.

When wounded or scratched, cell monolayers respond to the disruption of their cell to cell contacts with either apoptosis or a combination of proliferation and migration. This seems to depend on cell line sensitivity and the concentration of growth factors and cytokines at the wound margin (Firth and Putnins, 2004; Coomber and Gotlich, 1990; Zahm *et al*., 1997). Apoptosis in response to a scratch wound was reported in an epidermal keratinocyte cell line, and this effect was exacerbated by the presence of TNFα (Firth and Putnins, 2004). As shown in these results, in cultures with serum-deprived media, SW756 cells did not respond to the scratch wound with apoptosis, as confirmed by trypan blue exclusion assay, but with unidirectional and synchronized

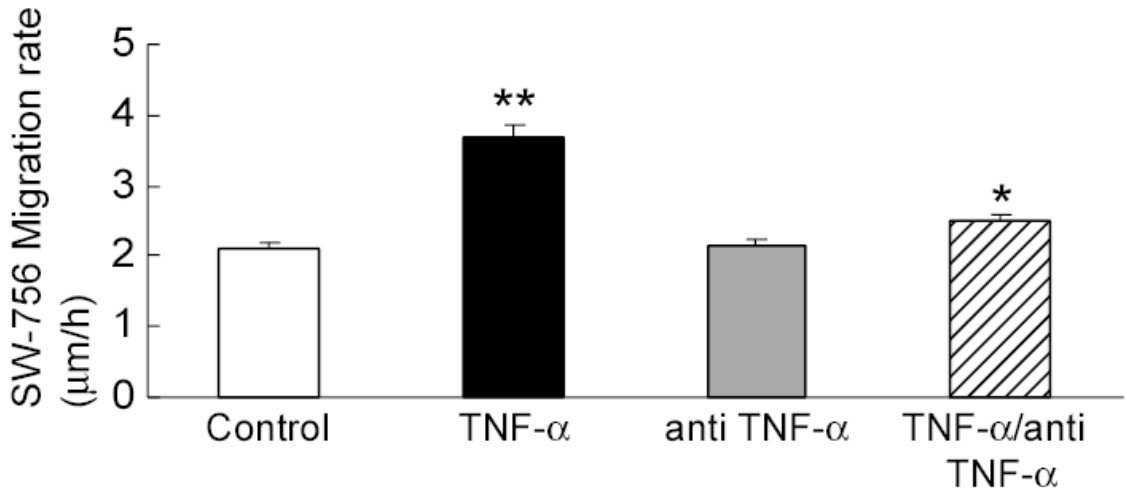

FIGURE 5. TNFα increases the migration rate of SW756 cells. The rate of migration (μm/h) of SW756 cells into the wound space was evaluated as half the distance difference between wound edges at 0 and 24 h, divided by 24. The distance between the edges was monitored by digital photographs taken in a phase-contrast microscope across the wound at the moment of wounding and at 24 hrs post-wounding. Images were analysed using an Image Pro-Express software 4.01. Results are the mean ± SEM of three independent experiments performed in triplicate.
*P<0.001 for TNFα as compared to control, antiTNFα, and anti-TNF/TNFα (Wilcoxon and Kruskal Wallis).
** P<0.02 for TNFα/antiTNFα as compared to control (Wilcoxon and Kruskal Wallis).

migration towards the empty wound space. This agrees with previous reports in which human ovarian (SK-OV-3 and CaOV-3) and cervical (SiHa and HT-3) carcinoma cell lines were resistant to the cytolytic action of TNFα (Powell *et al*., 1998). Furthermore, in more recent studies it was shown that human gastric MKN45 and cervical HeLa carcinoma cells could become susceptible to the apoptotic effects of TNFα by a combination of TNFα and Sulindac, an inhibitor of NF-κB (Yasui *et al*., 2003).

Stimulation of cell migration in an *in vitro* wound healing assay is complex, and requires the coordination of numerous events. Several endogenous, as well as, exogenous factors have been implicated in cell migration. Reorganization of the actin cytoskeleton (Lauffenburger and Horwitz, 1996) as well as activation of myosin II chain play important roles in changes of cell polarity and directionality during migration (Fumoto *et al*., 2003). The mechanisms by which TNFα could mediate cell migration have not been elucidated. TNFα activation of the transcription factor NF-κB seems to be critical for smooth muscle cell migration (Wang *et al*., 2001) and also for its mitogenic effect (Peppel *et al*., 2005). However, TNFα induced mitogenesis requires co-stimulation with other growth factors (Peppel *et al*., 2005). Therefore, in a cell culture exposed to serum-deprived media, TNFα could induce migration, but not cell proliferation due to the lack of additional growth factors present in the serum. This was confirmed in these studies, where serum-starvation of SW756 cultures resulted in inhibition of cell proliferation without loss of the ability of the cells to migrate in response to a scratch wound.

The present study showed a stimulatory effect of exogenous TNFα on migration of the cervical carcinoma cell line SW756. Since carcinomas, an in particular cervical carcinoma, are infiltrated by high amounts of stromal cells with the ability to secrete TNFα, such as mast cells and macrophages (Rojas *et al*., 2005; Cabanillas *et al*., 2002; Coussens and Werb, 2001), increased TNFα production at the tumor stroma could promote cancer cell migration, and therefore tumor invasion and metastasis.

Acknowledgements

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