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Research Article

Divergent effects of the major mast cell products histamine, tryptase and TNF-alpha on human fibroblast behaviour

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Abstract. Fibroblast proliferation is a key process in tissue remodeling and mast cells (MCs) are thought to play a crucial role. Having established that the three major MC products, tryptase, histamine and TNF-alpha (TNF) are normally present in human skin MCs, which are in close proximity to dermal fibroblasts, we studied their individual effects on cell cycle-controlled human dermal fibroblasts (HFFF2). These cells express receptors (H1, PAR2, TNFR1/2) for the major MC mediators, but only tryptase or a PAR2 agonist peptide stimulated proliferation and gene expression. TNF was antimitotic, and histamine, while elevating intracellular Ca^{2+} levels at high concentrations, did not affect proliferation. We conclude that MC products but also composition and numbers of respective receptors on fibroblasts are crucially responsible for fibroproliferative events.

Key words. Mast cell; histamine; tryptase; TNF-alpha; tissue remodeling; proliferation; calcium.

Introduction

Connective tissue remodeling processes are observed in a variety of organs including the testes [1, 2], for example, as a consequence of several allergic conditions [3], inflammatory reactions and after exposure to ionizing radiation [4]. One major step underlying fibroproliferative tissue responses is known to be fibroblast proliferation [5–7].
Mast cells (MCs), by secreting a plethora of mediators (e. g. proteases, cytokines and prostaglandins) appear to be direct players in tissue reorganization [8], wound healing [9] and cell proliferation [6, 10]. Several studies employing MC sonicates [7, 11], MC/fibroblast cocultures [11, 12] and immunoglobulin (Ig) E-activated MCs [13, 14] showed that functionally active human MCs stimulate proliferation of fibroblasts. MC-derived products are mainly stored in intracellular vesicles and are secreted upon MC activation [10, 15, 16]. Histamine (H), tryptase (T) and TNF-alpha (TNF) represent the major preformed MC secretory mediators stored in MC granules, and up to 10 pg T and up to 8 pg H are reported to be contained in a single human MC [10]. It is thus conceivable that after MC degranulation, H, T and TNF may have a vast impact on nearby cells (e. g. fibroblasts), assuming that these bear the respective receptors. Indeed, several authors by studying a variety of different *in vitro* systems have described a highly variable pattern of actions of MC-derived products on cell proliferation of fibroblasts, suggesting that the proliferative outcome might be highly dependent on parameters such as origin of the target cells, stage of cell cycle, receptor composition and also

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the ratio of MC-derived mediators and their coordinated release [2, 3, 5, 7, 17–27].

After ensuring that H, T and TNF are normally contained in MCs of the human skin, we showed divergent effects of these major MC products on proliferation and early signal transduction events of a cell cycle controlled human fibroblast model bearing receptors for H, T and TNF. We propose that fibroblastic tissue remodeling and wound healing processes are mainly regulated by the amount of T and TNF released from dermal mast cells and by the composition and number of the respective receptors on fibroblasts.

Materials and Methods

Skin samples, immunohistochemistry, TUNEL apoptosis labeling. Human skin tissue samples of healthy donors obtained from the archives of the Department of Dermatology and Allergy were fixed and embedded in paraffin. All participants granted written informed consent. The study was approved by the local Ethical Committee.

As previously described [28], deparaffinized tissue sections of human skin were treated with 3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity and were then incubated with 5% normal goat serum for 30 min to prevent non-specific antibody binding. The sections were incubated overnight at 4 °C with a monoclonal anti-human mast cell tryptase antibody (dilution 1:50, DAKO, Hamburg, Germany), a polyclonal anti-human histidine decarboxylase (HDC) antibody (dilution 1:200, Eurodiagnostica Arnhem, Netherlands) or a monoclonal anti-human TNF-alpha antibody (dilution 1:200, Chemicon, Hampshire, UK) and were probed with a biotin-coupled goat anti-mouse antibody (1:500) and a biotin-coupled goat anti-rabbit antibody (1:500), respectively. The sites of immunoreaction were visualized by the ABC method (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) and addition of 3,3'-diaminobenzidine tetrahydrochloride solution containing H_2O_2. Controls consisted of non-immune rabbit or mouse normal serum (1:5000) or omission of the primary antibody. For TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) apoptosis labeling, cells were washed three times in PBS, fixed in 4% paraformaldehyde for 30 min, washed twice in PBS and dried at room temperature for 30 min. TUNEL stainings were performed using the in situ cell death detection, POD according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

Cell culture. hFFF2 (human fetal foreskin fibroblasts; European Collection of Cell Cultures, Salisbury, UK) were maintained in DMEM supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich Chemie GmbH, Schellendorf, Germany). Cell cycle characteristics of hFFF2 cells were previously reported [29]. For proliferation assays, cells were incubated in FCS-free medium for 24 h to synchronize the cell cycle. To drive G0 cells into cell cycle, we treated serum-starved hFFF2 with medium containing 2.5% FCS. After 20 h, when cells entered into S phase, they were incubated in the presence or absence of H (Sigma-Aldrich), T (recombinant human skin tryptase, Promega, Madison, WI, USA), SLIGKV peptide (SLIGKV-amide, NeoMPS, Strasbourg, France) or TNF (recombinant human TNF-alpha, Sigma-Aldrich). For all other experiments, hFFF2 cells were cultivated in DMEM supplemented with 10% FCS.

RT and PCR analysis. RNA extraction was performed using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany), followed by reverse transcription (RT) using oligo-dT_{15} or random hexamer primers and polymerase chain reaction (PCR) amplification [30]. The following primers were used:

Histamine-1 receptor (H1): 5'-CTACAAGGCCGTACGACA-3' and 5'-CCTGCTCATCTGTCTTGA-3' yielding a 371-bp fragment. Histamine-2 receptor (H2): 5'-TCTACCGCATGCAAGATC-3' and 5'-CGAGGCTGATCATGAAGA-3' in combination with the following nested primers: 5'-TCATCCTCATCACCGTTG-3 and 5'-TGGTAGATGGCAGAGAAG-3', yielding a 155-bp fragment. Histamine-3 receptor (H3): 5'-ATGTACCCTACGTGCTGA-3' and 5'-GTGATGAGGAAGTACCAG-3' in combination with the following nested primers: 5'-CAACATCGTGCTCATCAG-3 and 5'-TACTCCCAGCTCAGGATG-3', yielding a 158-bp fragment. Histamine-4 receptor (H4): 5'-TCTCAGTAGGTGCCAAAG-3' and 5'-AGAATGGCCAGTGACTTG-3' in combination with the following nested primers: 5'-GAGACAGAGGAGAAAGAG-3 and 5'-GGCTCTAAGCAGTTCAAC-3', yielding a 142-bp fragment. Protease-activated receptor-2 (PAR2): 5'-CATCCTGCTAGCAGCCTC-3' and 5'-ACCTCTGCACACTGAGGC-3'; yielding a 480-bp fragment. Tumor necrosis factor receptor-1 (TNFR1): 5'-ACCGGCATTATTGGAGTGAAAA-3' and 5'-GGGGTAGGCACAACTTCGTG-3' yielding a 198-bp fragment. Tumor necrosis factor receptor-2 (TNFR2): 5'-CGCTCTTCCAGTTGGACTGAT-3' and 5'-CACAAGGGCTTCTTTTTCACCT-3' yielding a 105 bp fragment. Genomic DNA isolated from the H295 adrenocortical cell line or tissue library complementary DNA (cDNA) was used as positive control in all PCR experiments. Negative controls were performed by omitting the respective cDNA. The identity of PCR products was verified by commercial sequencing with a fluorescence-based dideoxy sequencing reaction and an automated sequence analysis.

Proliferation assays. Between 5000 and 10,000 hFFF2 cells were plated per well of a 96-well plate (Nunc GmbH & Co. KG, Wiesbaden, Germany), cell cycle synchronized and incubated in the presence or absence of different concentrations of H, T, TNF or PAR2 agonist peptide SLIGKV. Cell proliferation was determined by using the CellTiter 96 Aqueous One Solution cell-proliferation assay (Promega GmbH, Mannheim, Germany) after 24 h of stimulation. Experiments were performed three times independently with at least duplicate samples.

Calcium measurements. For Ca^{2+} measurements, hFFF2 were grown on coverslips for 24 h in DMEM supplemented with 10% FCS. The cells were loaded with 5 µM fluo-4, acetoxy-methylester (AM) (Molecular Probes, Eugene, OR, USA) in DMEM (+ 10% FCS) for 30 min at 37 °C and 5% CO_2. Finally, the cells were washed with FCS-free DMEM and transferred into a recording chamber mounted on a TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence was monitored at 500–540 nm (λ_{ex} = 488 nm) every 2s and the intensity was quantified over single cells. Real-time changes of intracellular Ca^{2+} levels were recorded during application of 10 µM H , 100 µM H, 100 µM pyrilamine (RBI, Natick, MA, USA), 100 µM H + 100 µM pyrilamine, 10 µM SLIGKV, 1 µg/ml T or 100 ng/ml TNF [31].

Gene arrays. Gene expression was evaluated in hFFF2 cells incubated for 60 min in the presence or absence of 100 ng/ml T, using the commercial chemiluminescent human signal transduction gene array kit (SuperArray, Biomol GmbH, Hamburg, Germany). For the respective membrane, mean background intensity (PUC18 DNA) was substracted from the intensity of each gene and signal intensities were normalized to the mean intensity of the housekeeping gene peptidylprolyl isomerase A (PPIA). Only genes with an expression level ≥ 10% PPIA were further quantified. Gene expression ratios were calculated by dividing the standardized density of the T-treated samples by the standardized density of the non-treated samples and expressed in fold up- or down-regulation.

Transmission electron microscopy (TEM). For ultrastructural studies hFFF2 cells were incubated with or without the respective stimulant and then fixed with 4% paraformaldehyde/0.5% glutaraldehyde and post-fixed with 4% OsO_4/potassium hexacyanoferrate (II). After embedding in Epon, semi-thin and ultra-thin sections were cut, contrasted with uranylacetate (2%)/lead citrate (2.7%) as described [32, 33] and examined with an EM10 electron microscope (Zeiss, Jena, Germany).

Data analysis and statistics. Data acquisition, analysis and statistics were performed using PRISM 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical analysis was performed utilizing the Kruskal-Wallis or Repeated measures ANOVA (analysis variance) test. Differences between the groups were evaluated using the appropriate post-test (Dunns or Newman-Keuls). Data represent the mean ± standard error of the mean, SEM.

Results

Human dermal mast cells contain T, TNF and HDC. Initially, in normal human skin, MCs were immunhistochemically identified by staining for T (fig. 1A), which is known to be contained in all human MC populations [10]. As shown in figure 1A, MCs are located in the papillary dermis in close proximity to dermal fibroblasts. Using serial tissue sections, we showed that T positive MCs also contain HDC, the rate-limiting enzyme in H synthesis (fig. 1B and C). In addition to T and HDC, some of the dermal MCs were also positive for TNF (fig. 1D). All controls performed by omitting the primary antibody or using non-immune rabbit or mouse serum were negative. Figure 1E displays a respective negative control.

Human fibroblasts cultured in vitro express receptors for H (H1), T (PAR2) and TNF (TNFR1 and TNFR2). To evaluate whether fibroblasts are potential target cells for MC-derived H, T and TNF, we performed RT-PCR experiments and detected the expression of the respective receptors in hFFF2 cells. Signals were found for the receptors H1, PAR2 and TNFR1. Low amounts of TNFR2 messenger RNA (mRNA) were also detected to be expressed by human fibroblasts (fig. 2).

Proliferation of human fibroblasts is enhanced by T and decreased by TNF, whereas H has no effect. Cell proliferation is a key step underlying tissue remodeling and wound healing processes. To evaluate the effect of T, TNF and H on proliferation of human fibroblasts, we utilized a cell culture model consisting of well-characterized primary hFFF2 cells, which were serum starved and driven into G1 20 h prior to stimulation, ensuring that the majority of cells were within the same stage of the cell cycle [29].

We determined the mitogenic potential of T, TNF, H and a PAR2 agonist peptide (SLIGKV) on hFFF2 cells using colorimetric cell proliferation assays. Many MC mediators are released in a coordinated fashion [15, 20]. Depending on the modus of mediator release, the amount of released mediator, the number and combination of respective receptors on the target cell and the velocity of mediator diffusion in the tissue, as well as mediator stability, the target cells might not encounter the whole spectrum of released MC products at the same time. Therefore, we investigated the separated effects of the

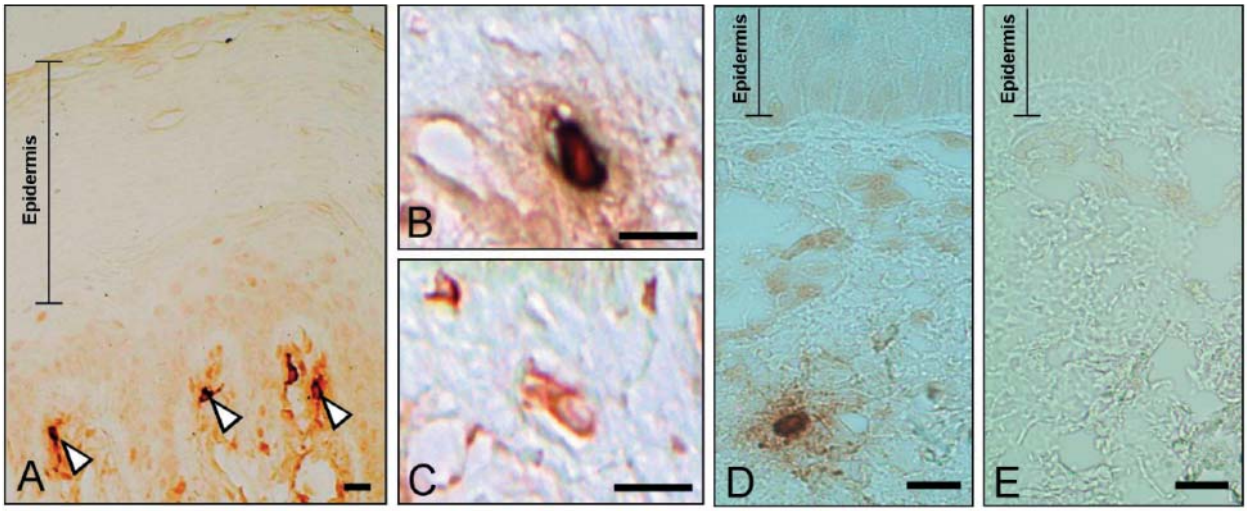

Figure 1. Dermal MCs express T, HDC and TNF. (*A*) Immunohistochemical staining for T, a protease known to be contained in all MC populations. T-positive MCs (arrowheads) are found underneath the epidermis in close proximity to dermal fibroblasts. (*B*) Magnification of a T-positive dermal MC. (*C*) Staining for HDC of the same MC located in a consecutive section. (*D*) Localization of a TNF-positive MC in human dermis. (*E*) Negative control performed by omitting the primary antibody. Bar, 15 µm.

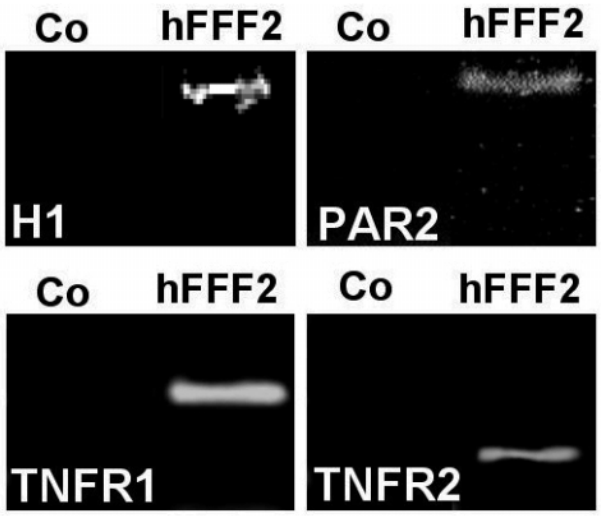

Figure 2. Human fibroblasts express receptors for H, T and TNF. RT-PCR experiments were performed using primers for the receptors H1, PAR2, TNFR1 and TNFR2 (for details see 'Materials and Methods' section). All receptors are expressed in hFFF2 cells. Co, negative control.

three major MC products on proliferation of hFFF2 cells. As shown in figure 3, H had no effect on proliferation of human fibroblasts (A). TNF reduced the number of hFFF2 cells in culture significantly in a dose-dependent manner after 24 h compared with the unstimulated control (B). In contrast, T was highly mitogenic and increased cell proliferation of hFFF2 cells up to three-fold in a dose-dependent fashion (C). We also used SLIGKV, which mimics the tethered ligand generated upon PAR2 cleavage by T. The proliferative effect of SLIGKV (C) was comparable to that induced by T.

The proliferative effect of T on human fibroblasts was also reflected at the molecular level. Signal transduction cDNA arrays performed with hFFF2 cells stimulated with T for 60 min indicated differential regulation of genes associated with cell proliferation. The expression of cyclin-dependent kinase inhibitors 1A (Genebank accession number L47233) and 2B (Genebank accession number L36844) was decreased in T-stimulated cells by about 20 and 62%, respectively (data not shown). A remarkable effect of T was also noted on mRNA expression of cyclooxygenase 2 (COX2), the key enzyme in prostaglandin synthesis. T enhanced the expression by 287% compared with the unstimulated control (data not shown). These results are in agreement with our previous observation that fibroblast proliferation is mediated by COX2-initiated generation and subsequent action of 15-deoxy-prostaglandin J2 [2] and confirm the PAR2-mediated mitogenic effect of T on human fibroblast.

H and TNF do not induce apoptosis in human fibroblasts. To address the possibility that the reduction in cell number 24 h after TNF stimulation was due to apoptosis, we studied the cellular and subcellular morphology of hFFF2 cells as well as the occurrence of DNA strand breaks related to apoptosis by TUNEL labeling. To explore the option that the unresponsiveness of hFFF2 cells to H regarding cell proliferation was due to apoptosis of a subset of fibroblasts, we also included H-stimulated cells into the experiment. Long term incubation (72 h) of hFFF2 cells with TNF (100 ng/ml) and H (100 µM) did not change the morphological appearance (fig. 4A) nor

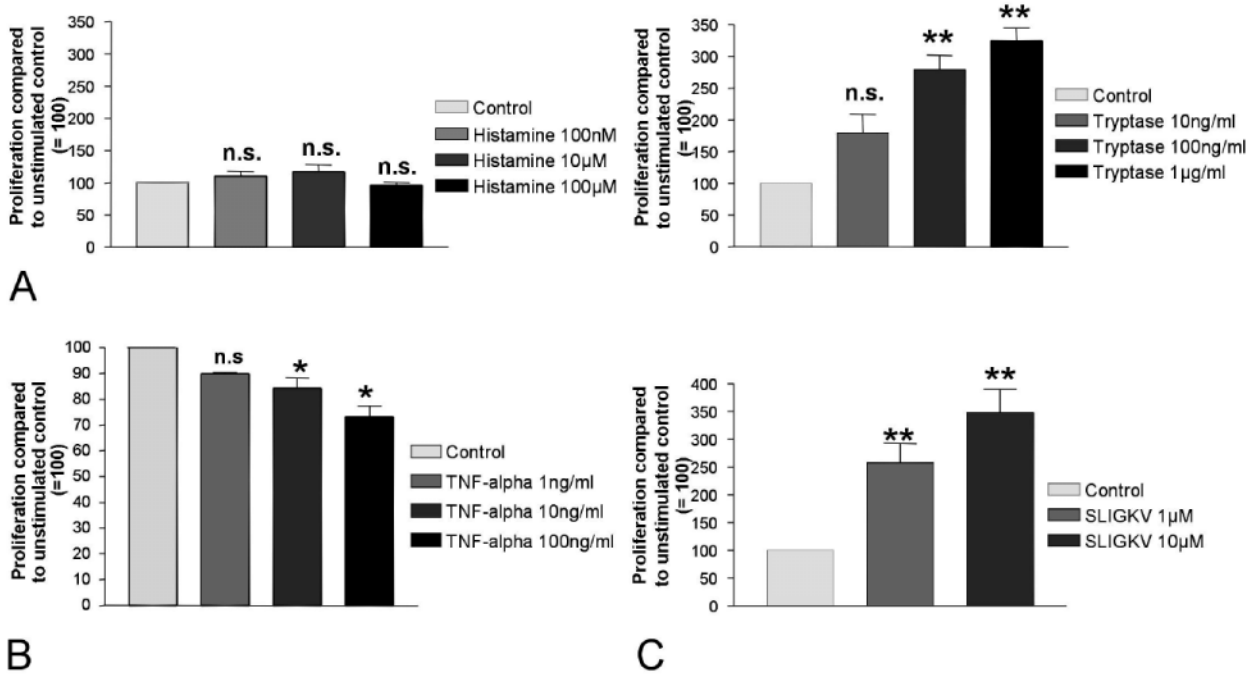

Figure 3. H, TNF, T and PAR2 agonist peptide SLIGKV exert diverse effects on proliferation of human fibroblasts. HFFF2 cells were incubated with H (100 nM, 10 µM, 100 µM; (*C*), TNF (1 ng/ml, 10 ng/ml, 100 ng/ml; T (10 ng/ml, 100 ng/ml, 1 µg/ml; or PAR2 agonist peptide SLIGKV (1 µM, 10 µM; for 24 h. Cell proliferation was measured using a colorimetric assay (for details see 'Materials and Methods' section). H has no effect on fibroblast proliferation (*A*), whereas TNF leads to a decrease in total cell number after 24 h (*B*). T and SLIGKV exert a dose-dependent mitogenic effect on human fibroblasts (*C*). All experiments were carried out three times independently with at least duplicate samples. Bars denote SEM; n. s., statistically not significant vs. control; asterisks (*) and (**) denote $P < 0.05$ and 0.001 vs. control.

did it induce any subcellular changes (data not shown). TUNEL stainings for apoptotic cells revealed no increased induction of apoptosis by H or TNF after 6 h and 24 h, respectively (fig. 4B).

In human fibroblasts, H1 and PAR2 but not TNFR1 or TNFR2 are associated with signal transduction mechanisms employing intracellular Ca^{2+}. In fibroblasts, signal transduction events initiated by H1 typically involve Ca^{2+} [22, 34]. In an attempt to examine the functionality of H1 on hFFF2 cells, we determined changes in intracellular calcium (iCa^{2+}) levels, using the Ca^{2+}-sensitive dye fluo-4 in combination with confocal microscopy. Stimulating hFFF2 cells with 100 µM H resulted in a transient increase in iCa^{2+} levels. This effect was completely blocked by the H1 antagonist pyrilamine (100 µM; fig. 5A). Lower concentrations of H (10 µM) did not induce changes in iCa^{2+} levels (fig. 5A inset).

Effects of TNF are mediated via the receptors TNFR1 and TNFR2 and complex intracellular signaling cascades [35, 36]. Although the respective receptors TNFR1 and TNFR2 are expressed by hFFF2 cells (fig. 2), TNF at concentrations clearly reducing the number of fibroblasts in culture after 24 h (fig. 3B, fig. 4) did not induce immediate changes in iCa^{2+} (fig. 5B). Early TNF-induced signal transduction events in human fibroblasts are therefore not coupled to an increase in iCa^{2+} levels.

Regarding PAR2, we have recently described that signal transduction events of PAR2 activate the MAP-kinase cascade, but are independent of Ca^{2+} as second messenger [37]. Nevertheless, here we show that at least a subset of fibroblasts (30–50%) is able to respond to the PAR2 agonist peptide (SLIGKV, 10 µM) with an increase in iCa^{2+} levels (fig. 5C). In the majority of the cases in which SLIGKV provoked iCa^{2+} fluxes in hFFF2 cells, subsequently applied T (1 µg/ml) was not able to increase iCa^{2+} levels, pointing towards a desensitization of PAR2 action by SLIGKV (fig. 5C). Nevertheless, 10% of the desensitized cells responded to subsequent stimulation with T (1 µg/ml) with an increase iCa^{2+} levels (data not shown).

Discussion

The involvement and importance of MC products in tissue remodeling processes has been well described by several authors in recent years [6, 10]. Nevertheless, there is still an apparent lack of information about the effects induced by different MC mediators in a defined, cell

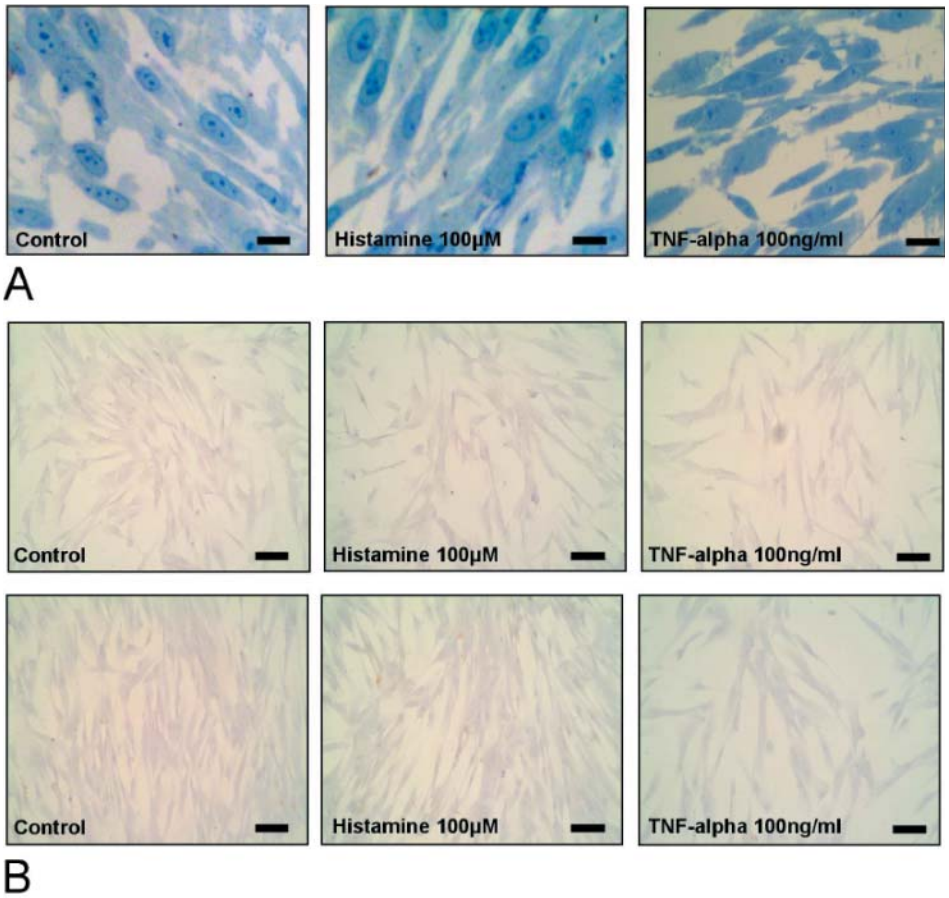

Figure 4. H and TNF do not induce apoptosis in human fibroblasts. Toluidine-blue stained semi-thin sections (*A*) and TUNEL labeling for apoptosis (*B*) of hFFF2 cells stimulated with H and TNF. Incubating the cells with the highest dose of H and TNF that influenced iCa^{2+} levels or numbers of hFFF2 cells does not lead to visible sights of apoptotic events after 72 h (*A*). There was also no induction of DNA strand breaks, indicative of apoptotic events, visible after H and TNF stimulation for 6 h (upper panel) or 24 h (lower panel) (*B*). (*A*) Bar, 10 µm. (*B*) Bar, 30 µm.

cycle controlled culture system, evidenced for instance by contradictory results that have been published on the proliferative potential of H, TNF and T [2, 3, 5, 7, 17–25, 36, 38–40]. After their release, MC products can interact with receptor-bearing target cells if these are in close proximity. Indeed our immunohistochemistry experiments showed that MCs immunoreactive for T, TNF and HDC, the rate limiting enzyme in H synthesis, are located nearby dermal fibroblasts in human skin. Upon activation by various stimuli [4, 41–43] MCs are therefore likely to influence fibroblast behaviour in a paracrine fashion and can thus be assumed to be involved in tissue remodeling processes [8, 9]. Since fibroblast proliferation is a hallmark of connective tissue reorganization, wound healing and fibrosis, we considered an *in vitro* system of human fibroblasts to be most suited to elucidate acute effects of the MC mediators H, TNF and T on cell proliferation. We selected a well-characterized cell culture model of primary human fibroblasts (hFFF2), which we proved to express receptors for H (H1), T (PAR2) and TNF (TNFR1 and TNFR2) and for which cell cycle dynamics were described previously [29]. Serum-starved fibroblasts were driven into G1 20 h prior to stimulation, ensuring that the majority of cells were within the same stage of the cell cycle.

The results of our study show that the three major MC products exert distinctively different effects on fibroblast proliferation, one of the key events in tissue reorganization and wound healing. T and a specific PAR2 agonist peptide are highly mitogenic, whereas TNF reduces the number of cells significantly and H has no effect on cell proliferation of fibroblasts after 24 h.

Concerning the effect of T on fibroblast proliferation, the vast majority of reports point to a mitogenic influence of this typical MC product [2, 5, 7, 44, 45]. This has also been confirmed by the outcome of our study that showed a significant increase in fibroblast proliferation after stimulation with T. A similar effect was observed after treating the cells with a PAR2 agonist peptide (SLIGKV).

Whether the activation of PAR2 due to enzymatic cleavage of the receptor by T is the only inducer of PAR2-mediated signal transduction events remains to be shown. Results of 'desensitization experiments' performed with combinations of SLIGKV and T showed that most but not all of the cells could be desensitized by pre-incubation with SLIGKV and therefore would argue for the above-mentioned action of T. However, we cannot exclude the possibility that T may influence fibroblast proliferation by cleavage of currently unknown receptors or

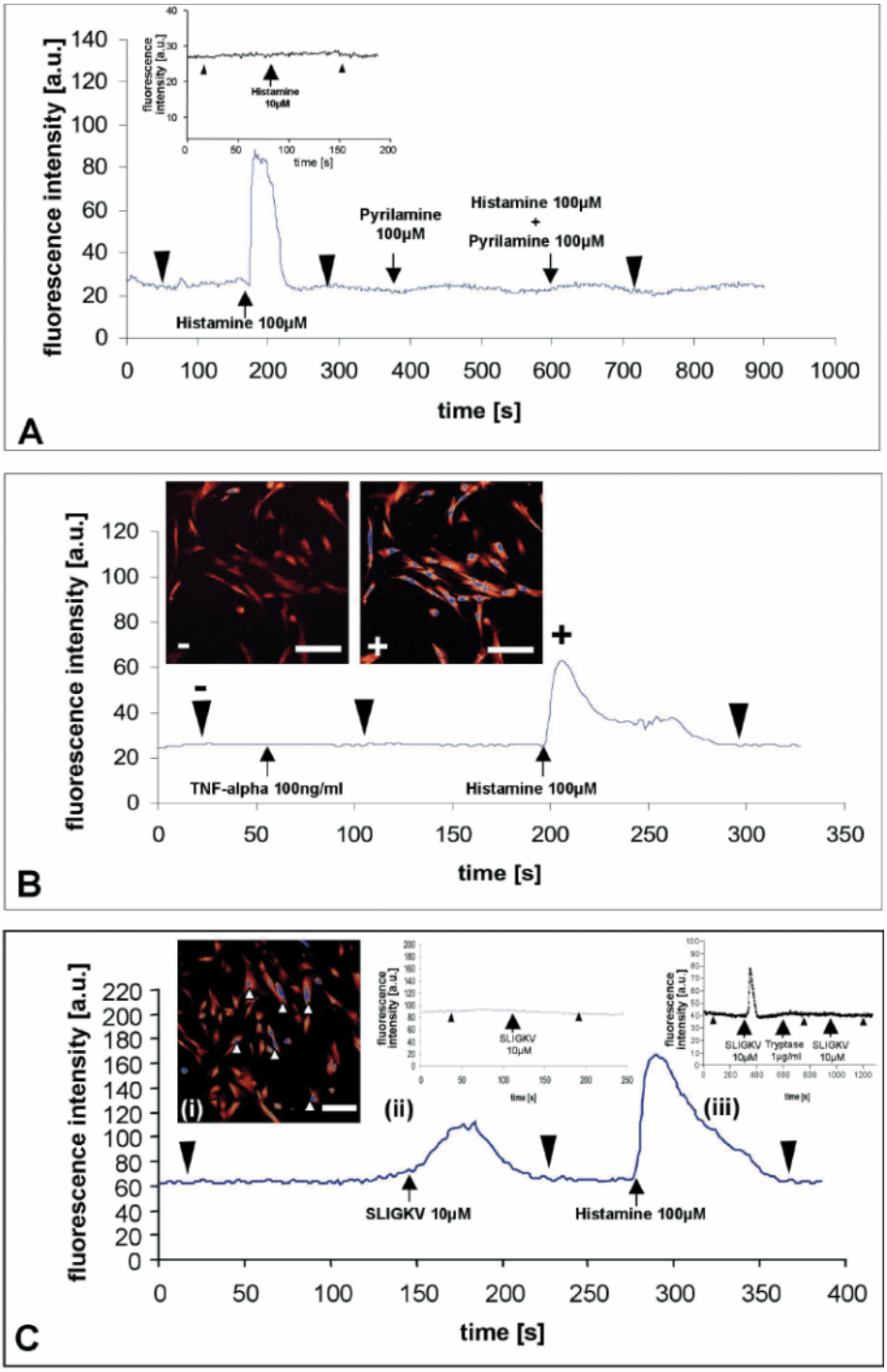

Figure 5. H increases intracellular Ca^{2+} (iCa^{2+}) levels were evaluated every 2 s. Time course of iCa^{2+} is shown for one representative cell levels via H1 receptors. TNF does not regulate intracellular Ca^{2+}, whereas the PAR2 agonist SLIGKV induces divergent changes in intracellular Ca^{2+} levels. HFFF2 were loaded with the Ca^{2+}-sensitive dye fluo-4, AM and treated with the respective stimulants. Intracellular Ca^{2+}. (*A*) H induces H1 receptor-mediated Ca^{2+} fluxes in human fibroblasts. An increase in iCa^{2+} was observed after addition of H (100 µM). This effect was completely blocked by the H1 antagonist pyrilamine (100 µM). 10 µM H had no effect on iCa^{2+} concentrations in hFFF2 cells (inset). All experiments were independently carried out 5 times, with 8–10 single cell determinations per experiment. (*B*) TNF at concentrations significantly reducing hFFF2 cell numbers (100 ng/ml) does not induce Ca^{2+} fluxes in human fibroblasts. An increase in iCa^{2+} was observed only after addition of H (100 µM) as positive control. Insets show respective fluorescence images taken after the addition of buffer (–) or H (+). Note that virtually all cells respond to H. All experiments were independently carried out 5 times, with 8–10 single cell determinations per experiment. Bar, 40 µm. (*C*) PAR2 agonist peptide (SLIGKV) at concentrations significantly increasing hFFF2 cell numbers (10 µM) leads to a diverse induction of Ca^{2+} fluxes in human fibroblasts. An increase in iCa^{2+} was observed after addition of SLIGKV and H (100 µM, positive control). As is shown in the insets (i and ii), not all human fibroblasts respond to SLIGKV with changes of iCa^{2+} levels. (i) Fluorescence image taken after SLIGKV (10 µM) addition. Responsive cells are denoted by arrowheads. (ii) Single cell measurement evaluating iCa^{2+} levels in one unresponsive hFFF2 cell. (iii) Representative desensitization experiment showing that pre-incubation of hFFF2 cells with SLIGKV (10 µM) renders the cell unresponsive to subsequent stimulation with T (1 µg/ml). All experiments were independently carried out 5 times, with 8–10 single cell determinations per experiment. Bar, 40 µm.

proteins and induction of signal transduction events utilizing iCa^{2+}. Interestingly, our gene array results showed a dramatic upregulation of COX2 expression following T stimulation in hFFF2 cells. COX2 is the inducible form of cyclooxygenase and a key enzyme in prostaglandin synthesis. We have recently shown that COX2 is involved in fibroblast proliferation via the production of 15-deoxy prostaglandin J2 and that inhibiting COX2

leads to a reduction of cell proliferation, whereas addition of 15-deoxy prostaglandin J2 has a mitogenic effect [2]. Therefore, we believe that COX2 plays a central role in T-induced cell proliferation of human fibroblasts.

Regarding the influence of H on cell proliferation, contradictory data have so far been published. This might at least partly be due to the fact that FCS contains unknown factors, including some H-degrading enzyme activity related to semicarbazide-sensitive amine oxidases. Our present results obtained in studies using culture media with FCS failed to show any influence of H or its possible metabolites on fibroblasts. In respect of proliferation our results are partially in line with the outcome of a study performed by Johnson et al. employing human skin fibroblasts. The authors revealed an inhibitory action of H on incorporation of 3H-thymidine into DNA, which was accompanied by an induction of inositol 1, 4, 5-triphosphate (InsP3) and a subsequent increase in iCa^{2+} levels [22, 47]. These effects were blocked by an H1 antagonist, and the authors suggested that H1 mediates a block of the cell cycle. Using primary human lung fibroblasts, Jordana et al. found a proliferative, H2 receptor-mediated effect of H. This effect only occurred when cells were exposed to H at restricted times during cell growth and was thus shown to depend in part on the stage of the cell cycle [17]. Our unpublished data showed the lack of H3 and H4 receptor expression in human fibroblasts, whereas H2 receptors could be detected by a nested PCR approach in hFFF2 cells. We conclude that if H2 receptors are present on hFFF2 cells at all, they will be expressed only in low quantities at the cell membrane, and therefore we do not expect H2-mediated signal transduction events to regulate proliferation of hFFF2 cells.

TNF can exert its functions via the two receptors TNFR1 and TNFR2, which both are expressed by hFFF2 cells. TNFR1 and TNFR2 contain an extracellular pre-ligand-binding assembly domain that pre-complexes receptors and encourages them to trimerize upon activation by TNF ligand [40]. Pleiotropic actions of TNF have been described that range from proliferative responses such as cell growth and differentiation, inflammatory effects and the mediation of immune responses, to destructive cellular outcomes such as apoptotic and necrotic cell death mechanisms [36]. The TNFR1 contains a so-called death domain region towards the carboxy-terminal end of the receptor. The death domain is also present on a number of associating proteins and related molecules that are involved in signaling for cell death [48]. Although, hFFF2 cells show a strong expression of TNFR1 mRNA and despite the fact that incubating the human fibroblasts with TNF resulted in a lower number of fibroblasts after 24 h when compared with untreated controls, we could not detect signs of necrosis or apoptotic events in the cultures by TUNEL assays or by assessing the cell morphology on semi-thin sections. Therefore, the effect of TNF on cell number of human fibroblasts appears to be due to cell cycle-based antimitogenic mechanisms.

Activated TNF receptors mediate the association of distinct adaptor proteins that regulate a variety of signaling processes, including kinase or phosphatase activation, lipase stimulation, and protease induction. Moreover, TNF influences transcription factors, heterotrimeric or monomeric G-proteins and calcium ion homeostasis in order to orchestrate cellular functions [36, 49]. In our study, we could not detect any clear influence of TNF on iCa^{2+} levels of human fibroblasts. These results are in line with findings that TNF itself does not alter iCa^{2+} levels but enhances the activity of iCa^{2+}-sensitizing agents [50, 51]. However, Kong et al. described a raise in iCa^{2+} levels of L929 fibroblasts which was only observed after prolonged TNF stimulation, inducing cytotoxic effects [52].

H-mediated signal transduction events in fibroblasts involving Ca^{2+} are characteristically related to H1 [22, 34]. To investigate the functionality of H1 on hFFF2 cells, we measured iCa^{2+} levels and found evidence for a rapid increase in iCa^{2+} levels following H stimulation. This effect was observed only when high concentrations of H (100 μM) were applied, but was completely blocked with an H1 antagonist, confirming the existence of functional H1 receptors on the surface of human fibroblasts, which in our case were not related to proliferative or apoptotic events.

PARs are physiologically activated by a proteolytic mechanism in which the respective protease (e. g. T) binds to and cleaves the amino-terminal domain of the receptor. This cleavage generates a new N-terminus which functions as tethered ligand, binding to the receptor and initiating transmembrane signaling events [53–55]. Activation of the respective receptor can also be achieved by employing PAR-specific agonist peptides, such as SLIGKV, which mimic the tethered ligand [54, 58, 59]. Inverting the first two amino acids of the tethered ligand (SLIGKV) to LSIGKV results in loss of function of this peptide, proving the high specificity of SLIGKV for PAR2 [60]. As we have recently described, treatment of hFFF2 with T or PAR2 activating peptide (SLIGKV) had failed to induce changes in iCa^{2+} levels, but led to phosphorylation of erk1/2 as well as increased expression of c-jun and c-fos [37]. Contrary to our previous data, here we show that a subset of hFFF2 fibroblasts responds with increased iCa^{2+} levels to PAR2 activation, whereas the partially inverted peptide had no effect (data not shown). The increase of iCa^{2+} levels was unexpected, and at present we do not have a proper explanation for these discrepancies obtained with the same experimental setup. We speculate, however, that – as hFFF2 cells are primary fibroblasts – differences in cultivation time might be related to the changed responsiveness to PAR2 activation.

In conclusion, our study is the first report evaluating in parallel the effects of H, TNF and T on a defined cell culture system of cell-cycle-controlled human fibroblasts. We found evidence that the MC mediators TNF and T act in a diverse manner on cell proliferation. In contrast to TNF, which acts antimitogenically, and T, which acts proliferatively on human fibroblasts via PAR2, H has no effect on proliferation. We propose that fibroblastic tissue remodeling and wound healing processes are mainly regulated, on the one hand, by the amounts of tryptase and TNF released from dermal mast cells, and on the other, by composition and number of the respective receptors on fibroblasts. Indeed, our unpublished immunohistochemical results show receptors for these typical MC products on dermal fibroblasts, pointing towards their participation in physiological and pathophysiological processes in human skin.

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