

# TGF $\beta$ Presence During IgE-dependent Sensitization Primes Mast Cells for Higher VEGF Production After Fc $\epsilon$ RI Activation

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**Abstract:** Binding of monomeric Immunoglobulin E (IgE) to the high affinity IgE receptor (Fc $\epsilon$ RI) on mast cells induces a sensitization process which increases cell survival, augments membrane receptor expression and diminishes activation threshold. Although IgE-dependent sensitization is fundamental for allergic reactions, little is known about the influence of locally produced mediators on the outcome of a posterior allergen challenge. Since Transforming Growth Factor  $\beta$  (TGF $\beta$ ) is an important immunomodulator present in most of the tissues where mast cells reside, we decided to analyze the consequences of TGF $\beta$  exposure during the sensitization step of mast cells on a posterior IgE-antigen stimulation. Bone Marrow-derived Mast Cells (BMMCs) were sensitized with IgE in the presence or absence of TGF $\beta$ . Then, antigen was added and the secretion of the angiogenic cytokine Vascular Endothelial Growth Factor (VEGF) was determined. BMMCs sensitized with IgE+TGF $\beta$  showed an increased antigen-induced VEGF secretion compared to those sensitized with IgE alone. Sensitization with IgE+TGF $\beta$  did not modify membrane Fc $\epsilon$ RI receptor expression neither altered antigen-induced degranulation of the cells. Although both IgE and IgE+TGF $\beta$  sensitized cells showed an increase in VEGF mRNA stabilization after antigen addition, VEGF mRNA half-life was longer in IgE+TGF $\beta$  sensitized cells. p38 MAPK inhibitor SB202196 blocked VEGF mRNA stabilization after antigen addition specially on IgE+TGF $\beta$  sensitized cells. These findings suggest that TGF $\beta$  presence during the sensitization phase of mast cells can induce modifications to the Fc $\epsilon$ RI signal transduction system, provoking increased VEGF mRNA stabilization and protein secretion after IgE-antigen stimulation through a p38 MAPK-dependent mechanism.

**Keywords:** TGF $\beta$ , Mast cell, IgE, VEGF, Sensitization.

## INTRODUCTION

Mast cells are important players in anaphylaxis and immunoglobulin E (IgE)-associated allergic diseases such as atopic asthma, allergic rhinitis and atopic dermatitis [1]. Allergic individuals produce IgE antibodies that have specificity for the antigens to which the subjects are sensitive, and those antibodies are bound to the membrane of mast cells due its interaction with the high affinity IgE receptors (Fc $\epsilon$ RI) [2]. In the absence of antigen, binding of monomeric IgE to Fc $\epsilon$ RI receptors produce a sensitization process that allows mast cells to increase survival, augment Fc $\epsilon$ RI receptor expression on the plasma membrane, and undergo degranulation at lower concentrations of antigen, releasing larger amounts of inflammatory mediators after IgE-antigen stimulation [3]. Long-lasting sensitization with monomeric IgE is a common condition in atopic individuals, where high concentrations of IgE in plasma have been proposed to induce increased mast cell numbers and promote responsiveness to very low amounts of antigen [4].

Once cell sensitization occurs, mast cells are ready to be activated by the antigen-mediated crosslinking of monomeric receptors. Activated Fc $\epsilon$ RI receptor turns on a complex signaling cascade, which include activation of at least two different Src-family kinases (Lyn and Fyn), activation of Syk kinase, phosphorylation of different adapters (such as LAT and Gab2) and the formation of multimolecular aggregates that can connect the initial signal to intracellular calcium rise, MAP kinase activation, cytoskeletal movements and differential gene expression [5]. After Fc $\epsilon$ RI crosslinking, mast cells are able to start the synthesis of multiple cytokine mRNAs that will be translated at different rates, allowing them to secrete a variety of interleukins, chemokines and angiogenic factors [1].

One of the more distinctive characteristics of the Fc $\epsilon$ RI signal transduction system is the capacity of coupling to specific cellular effectors depending on the particular composition of the multimolecular complexes formed at the plasma membrane [6], this important feature suggest that, potentially, signaling could be modulated by the presence of different locally-produced mediators surrounding mast cells. Those cytokines, growth factors or neurotransmitters, acting on mast cells, could alter the availability of signaling mole-

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cules in different complexes, or produce positive/negative regulators of the Fc $\epsilon$ RI signaling pathway [7].

TGF $\beta$ s are regulatory cytokines with pleiotropic effects on cell proliferation, differentiation, migration and survival, affecting multiple biological processes including development, fibrosis, wound healing, and immune responses [8]. There are three homologous TGF $\beta$  isoforms in mammals, TGF $\beta$ 1, TGF $\beta$ 2, and TGF $\beta$ 3, encoded by different genes [9]. TGF $\beta$ 1 is the predominant isoform expressed in the immune system, but all three isoforms have similar properties *in vitro*. It is locally produced in tissues where mast cells reside and is importantly synthesized in conditions of tissue remodeling and angiogenesis [10]. TGF $\beta$ 1, normally referred solely as TGF $\beta$ , is a profibrotic and immunomodulatory factor that, depending on the conditions, can act as a pro- or anti-inflammatory cytokine [8].

Vascular Endothelial Growth Factor, known alternatively as VEGF-A, is one of the most potent angiogenic cytokines described to date [11]. Murine VEGF is encoded by a single gene located on chromosome 17 and alternative splicing yields three predominant VEGF variants, including VEGF<sub>120</sub>, VEGF<sub>164</sub> and VEGF<sub>188</sub> (corresponding to the human homologues VEGF<sub>121</sub>, VEGF<sub>165</sub> and VEGF<sub>189</sub>, respectively). VEGF variants possess different bioavailabilities, due to differential affinities for heparin and for specific VEGF receptors. The shorter splice variants are soluble, while longer splice variants associate with extracellular matrix components, through its interaction with proteoglycans [11]. Mast cells are able to produce VEGF after Fc $\epsilon$ RI activation [12, 13]. Human mast cell lines constitutively express VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub> transcripts and an important secretion is observed after mast cell stimulation, being VEGF present in secretory granules [12]. In murine peritoneal mast cells, an important antigen-induced increase on VEGF secretion was observed after a prolonged treatment with monomeric IgE [13].

Mast cell-secreted VEGF is involved in angiogenesis [14], the passage of IgE through the aortic endothelial cell monolayer [15] and also provokes changes on vascular permeability during the inflammation process [16]. Accordingly to the notion of a positive influence of mast cell-produced VEGF on angiogenesis, an increased number of mast cells have been observed in various tumors, including those in the lung [17], larynx [18], kidney [19], breast [20], and others [21]. High mast cell numbers positively correlates with increased microvessel density and has been considered as a sign of poor prognosis [22]. Despite of those findings, the main effect of mast cell-secreted VEGF seems to be related to enhanced microvascular permeability, causing leakage of macromolecules through the vesiculo-vacuolar organelle in endothelial cells [23]. Importantly, VEGF secretion after mast cell activation has been associated, together with TGF $\beta$ , to lung tissue remodeling observed in chronic asthma [24], a condition where the formation of new blood vessels has been reported.

In the present study, the effect of TGF $\beta$  during the IgE-dependent sensitization of mast cells on a posterior Fc $\epsilon$ RI receptor activation was analyzed. Particularly, we focused our attention on the analysis of VEGF secretion after Fc $\epsilon$ RI crosslinking, since the molecular mechanisms involved in the release of angiogenic factors during allergic reactions have not been fully described.

## MATERIALS AND METHODS

### Mice

Four to eight weeks old 129S1/SvImJ mice (Jackson Laboratories Stock # 002448) were sacrificed by CO<sub>2</sub> inhalation and bone marrow was extracted from both tibias by injection of 3 ml of complete BMMC media (see following sections) directly in the hollow of the bones. All the experimental procedures using mice were approved by our Institute Committee for the Experimental Use and Animal Care, (CICUAL, Protocol # 383-07).

### Cell Culture

Bone marrow derived mast cells (BMMCs) were differentiated seeding bone marrow in complete BMMC media containing RPMI 1640 culture media with L-glutamine (Sigma Cat. # R4130), 20 ng/mL IL-3 (Preprotech Cat. # 213-13), 25 mM HEPES buffer (Sigma, Cat. #R4130), 10% Fetal Bovine Serum (Gibco, BRL Cat. # 261400779), 1X nonessential amino acids (100X stock, Biofluids, Cat. #33-100), 1 mM sodium pyruvate (100 mM stock, Biofluids, Cat. #33-100), 50  $\mu$ M  $\beta$ -mercaptoethanol (Sigma, Cat. # M6250) and 1U/mL penicillin-0.1 mg/mL streptomycin (stock 100U/ml penicillin-10 mg/mL streptomycin GIBCO, Cat. #10378-016). Cell cultures were grown at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>, changing media once per week. Four weeks later, expression of Fc $\epsilon$ RI receptor was assayed by flow cytometry, and only the cultures showing more than 95% positive cells were used for the experiments. Human Vascular Endothelial Cells were purchased to BD Biosciences (HUVEC-2, Cat. # 354115) and were maintained in MCDB-131 Complete Medium (Vec Technologies, Inc., Cat. # MCDB-131 AB).

### Flow Cytometry

Routine flow cytometry for detection of Fc $\epsilon$ RI receptor in mature cultures was performed incubating 2x10<sup>6</sup> mast cells for one hour with 300 ng/ml of a monoclonal anti-dinitrophenol (DNP)-IgE (Sigma, Cat. # D 8406), in staining buffer (PBS pH 7.4, 0.5% bovine serum albumin, BSA, and 0.05% sodium azide). After this incubation, cells were washed once and incubated in the presence of a FITC-labeled anti-IgE antibody (BD Biosciences Cat. #553415). After 30 min incubation, cells were washed again and suspended in staining buffer before flow cytometry analysis, that was performed using a FACScan (Becton Dickinson). For changes on Fc $\epsilon$ RI receptor expression after sensitization, the same FITC-labeled anti IgE ab was used.

### Apoptosis Detection Assay

Mast cell apoptosis was evaluated using an Annexin V kit (BD Biosciences Cat. # 556547) following the instructions provided by the manufacturer. Briefly, 2x10<sup>6</sup> mast cells were incubated overnight with 300 ng/ml of IgE (Sigma, Cat. # D 8406), or IgE+100 pM TGF $\beta$  (PeproTech, Cat. #100-21R). Cells were washed with PBS and then Annexin-V-FITC and/or propidium iodide (PI) were added. Cells were incubated for 15 min at RT in the dark, washed once and suspended in the provided buffer prior to flow cytometry analysis.

### Cell Sensitization and Stimulation

Mature BMMCs were collected by centrifugation and suspended at  $1 \times 10^6$  cells/mL on fresh complete BMMC media warmed at 37°C. For IgE-dependent sensitization, 300 ng/mL of the monoclonal anti dinitrophenol (DNP)-IgE (Sigma, Cat. # D 8406) were added to the media; in the case of IgE + TGF $\beta$  sensitization, 300 ng/mL IgE plus 100 pM of recombinant TGF $\beta$  (Preprotech Cat. # 100-21R, TGF- $\beta_1$ ) were added to the cells. Cells then were incubated for 18 hours at 37°C. After this sensitization step, cells were collected and suspended in cell culture media or Tyrode's/BSA buffer (see following section) at 37°C. For Fc $\epsilon$ RI stimulation, different amounts of the synthetic antigen DNP coupled to Human Serum Albumin (DNP-HSA, Sigma Cat # A-6661) were added. Depending on the parameter to be evaluated, reaction was stopped at optimal times and degranulation assays, RNA extractions or VEGF secretion assays were performed (see following sections).

### $\beta$ -hexosaminidase Activity

Degranulation was measured determining the amount of  $\beta$ -hexosaminidase activity in the supernatants of cells stimulated for 1 hour as it was described in the previous section. After sensitization, cells were collected and suspended in Tyrode's / BSA buffer (130 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.4 mM CaCl<sub>2</sub>, 5.6 mM glucose, 20 mM HEPES and 0.1% BSA) at 37°C. Aliquots of  $2 \times 10^6$  IgE-sensitized cells in 1 mL of Tyrode's buffer were then stimulated by the addition of different concentrations of DNP-HSA for one hour. After this time, cells were placed on ice for 10 minutes and centrifuged at 10,000 x g at 4°C. Supernatants were transferred to a new tube and pellets were solubilized with 1 mL of 0.05% Triton X-100 (Sigma Cat. # T 9284). Sixty microliters of the supernatants and solubilized pellets were incubated at 37°C together with 20  $\mu$ l of 0.1 M 4-nitrophenyl-N-acetyl- $\beta$ -D-glycosaminide (PNAG, Sigma, Cat. #N9376) prepared in 0.1M citrate buffer (pH 4.5, 0.1M citric acid and 0.1M sodium citrate). After 1 hour, the reaction was stopped by the addition of 120  $\mu$ l of a solution containing 0.1M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>. Light absorbance was measured at 405 nm in an ELISA plate reader. The amount of  $\beta$ -hexosaminidase release was determined by dividing the activity observed in the supernatant by the total activity of the enzyme, detected in the supernatant plus cell pellet.

### RNA Isolation and RT-PCR

After sensitization, cells were collected and suspended in Tyrode's / BSA at 37°C. Aliquots of  $2 \times 10^6$  IgE-sensitized cells in 1 mL of Tyrode's buffer were then stimulated by the addition of different concentrations of DNP-HSA for one hour. After this time, cells were placed on ice for 10 minutes and centrifuged at 10,000 x g at 4°C. Cell pellet was used for total RNA extraction using TRI-Reagent (Sigma, Cat. #T3934). RNA pellets were suspended in 50  $\mu$ l of RNA Secure solution (Ambion, Cat. #7010) and quantified by light absorption at 260 nm. First strand cDNA synthesis was performed using the First Strand Synthesis Kit from Invitrogen (Cat. #11904-018). Two microliters from each final reaction were used to amplify the murine isoforms of VEGF or the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control. Primers used for RT-PCR were the following: VEGF sense, 5'ACA TCT TCA AGC CGT CCT GTG TGC

3; antisense, 5'AAA TGG CGA ATC CAG TCC CAG GAG 3; GAPDH sense 5' TGA-AGG-TCG-GTG-TGA-ACG-GAT-TTG-GC 3 and antisense 5'CAT-GTA-GGC-CAT-GAG-GTC-CAC-CAC 3'. In the case of primers for VEGF, they were able to amplify the three main isoforms of VEGF (120, 164, and 188 aminoacids) expressed in mice [25]. Amplification conditions were standardized changing amounts of cDNA, oligonucleotide concentration, annealing temperature and the number of cycles starting from the protocol reported in Ref. 25. We found that the linear range of amplification of the three isoforms was between 24 to 32 cycles. 28 cycles were used to get the best results and good detection, running one fifth of the PCR reaction volume in each lane of agarose gels. PCR conditions were as follows: hot start at 94°C (5 min), denaturation at 94°C (1 min), annealing at 60°C (2 min 30 sec) and extension at 72°C (4 min) for 28 cycles. PCR products were separated by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining. A 100-bp DNA ladder was included every time as a reference marker. Densitometric analysis of band intensity was performed using an image analyzer (UVP BioImaging Systems, EpiChemi<sup>3</sup> Darkroom), and the LabWorks 4.0 Image Acquisition and Analysis Software (UVP BioImaging Systems).

### ELISA

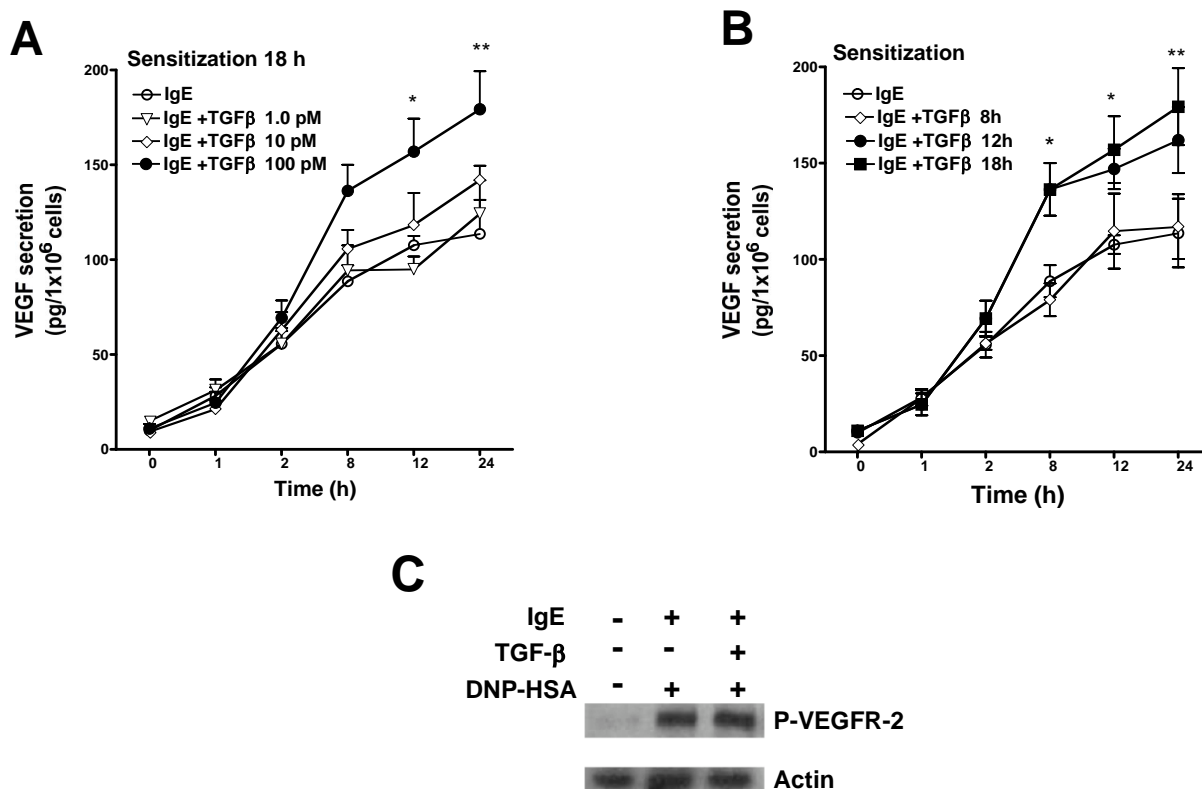
VEGF secretion after antigen stimulation of sensitized cells was analyzed using specific ELISA kits. After sensitization, cells were washed once and suspended in fresh media containing a protease inhibitor cocktail (Roche, Cat. #1836153, one tablet per 50 mL of media). Aliquots of  $5 \times 10^6$  cells were incubated at 37° for distinct times in the absence or presence of 27 ng/mL DNP-HSA. After each time, supernatants were collected and frozen. Secreted VEGF was analyzed with a mouse VEGF ELISA kit (Biosource International, Cat. # KMG0112) according to manufacturer's instructions. The limit of detection of the assay was 7.5 pg/ml. All measurements were performed by triplicate. This assay is reported to show no detectable cross-reactivity with a range of other growth factors and cytokines.

### Determination of VEGF Activity

Mast cell-derived VEGF activity was assayed by western blot, detecting the tyrosine phosphorylation VEGFR-2 receptor after mast cell supernatant addition to Human Vascular Endothelial Cells (HUVECs). Briefly, HUVEC-2 cells (BD Biosciences, Cat. # 354151) were incubated overnight in serum-free media. The next day, cells were plated in 12 well plates and incubated for 5 minutes with 0.1 mM Na<sub>3</sub>VO<sub>4</sub>. Then cells were stimulated for 5 minutes at 37°C with VEGF-containing supernatants obtained from stimulated mast cells, washed and suspended in lysis buffer (1% Triton X-100, 10mM Tris/HCl, pH 7.6, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride and 2  $\mu$ g/ml of aprotinin). Total protein was separated by SDS-PAGE. VEGFR-2 phosphorylated protein was detected using a monoclonal anti-phospho-VEGFR-2-Tyr 1175 (Cell Signaling Technologies 19A10 rabbit mAb, Cat. #24785).

### Statistical Analysis

All data shown are expressed as the mean  $\pm$  SEM, of at least three independent experiments. Statistical analysis was



**Fig. (1).** TGF $\beta$  presence during IgE-dependent sensitization of mast cells increases the release of active VEGF after Fc $\epsilon$ RI stimulation. Panel A. BMMCs were sensitized for 18 hours with IgE (300 ng/ml) alone or in combination with different concentrations of TGF $\beta$ . After this time, cells were washed once and suspended in fresh media containing 27 ng/ml of DNP-HSA. Supernatants were collected at different times of incubation at 37°C. VEGF concentration on the supernatants was determined by ELISA. Panel B. BMMCs were incubated in the presence of IgE (300 ng/mL). TGF $\beta$  (100 pM) was added at different times before the 18 hours-long sensitization process concluded. Cells were then collected and stimulated with 27 ng/ml of DNP-HSA at 37°C. Supernatants of antigen-activated cells were taken at different times after antigen addition. Data are expressed as the means  $\pm$  SEM from three different experiments using distinct cell cultures. \*  $p < 0.05$ ; \*\*  $p < 0.01$ , with respect to IgE-sensitized cells. Panel C. Supernatants containing 50 pg/ml of VEGF (measured by ELISA) were collected from IgE or IgE+TGF $\beta$ -sensitized cells after 8 hours of antigen stimulation. Supernatants were then added to HUVEC cells for 5 minutes. After this time, cells were lysed and phosphorylation of VEGFR2 was detected by western blot as described in Material and Methods section. A representative experiment (of three performed) is shown.

calculated using SigmaStat software (SYSTAT, 2004). Differences between groups were considered significant at  $p < 0.05$ .

## RESULTS

### TGF $\beta$ Presence During Mast Cell Sensitization Increases the Secretion of Functional VEGF after Fc $\epsilon$ RI Receptor Crosslinking

The influence of TGF $\beta$  during the sensitization step on the VEGF secretion elicited by Fc $\epsilon$ RI receptor activation was analyzed by incubating BMMCs for 18 hours in the presence of a monoclonal anti-dinitrophenol (DNP) immunoglobulin E (IgE), or with IgE plus different concentrations of TGF $\beta$ . After sensitization, cells were stimulated with the artificial antigen DNP-HSA and VEGF secretion at different time points was measured using a specific ELISA kit. As is shown in Fig. (1A), VEGF accumulation was maximal between 8 and 12 hours after antigen-stimulation of BMMCs and the presence of 100 pM TGF $\beta$  induced a significant increase on VEGF secretion after Fc $\epsilon$ RI triggering (50% increase over the observed value in IgE-sensitized cells at 12 h). This increased VEGF secretion

was evident even at 24 hours after antigen addition. In order to find the time necessary to observe the effect of TGF $\beta$ , BMMCs were sensitized with IgE for 18 h or with IgE plus TGF $\beta$  added at different times prior to antigen stimulation. As can be observed in Fig. (1B), it was necessary the presence of TGF $\beta$  for 12 to 18 hours prior to antigen stimulation to observe the increase on VEGF secretion. VEGF produced by IgE or IgE+TGF $\beta$ -sensitized cells was equally potent to provoke phosphorylation of VEGFR2 in HUVEC cells (Fig. 1C). This phosphorylation was prevented by the addition of a neutralizing antibody against VEGF to mast cells supernatants prior to HUVEC stimulation (data not shown).

### TGF $\beta$ Presence During Mast Cell Sensitization Does Not alter the Apoptotic Rate of BMMCs or the IgE-Dependent up Regulation of Fc $\epsilon$ RI Receptor Expression in Plasma Membrane

Since it has been shown that TGF $\beta$  can influence cell proliferation in different cell types [26], we investigated if the sensitization with IgE+TGF $\beta$  could modify mast cell apoptosis. We sensitized mature BMMCs cells with IgE or IgE+TGF $\beta$  for 18 hours and the apoptotic populations in the culture were detected by the expression of annexin V and

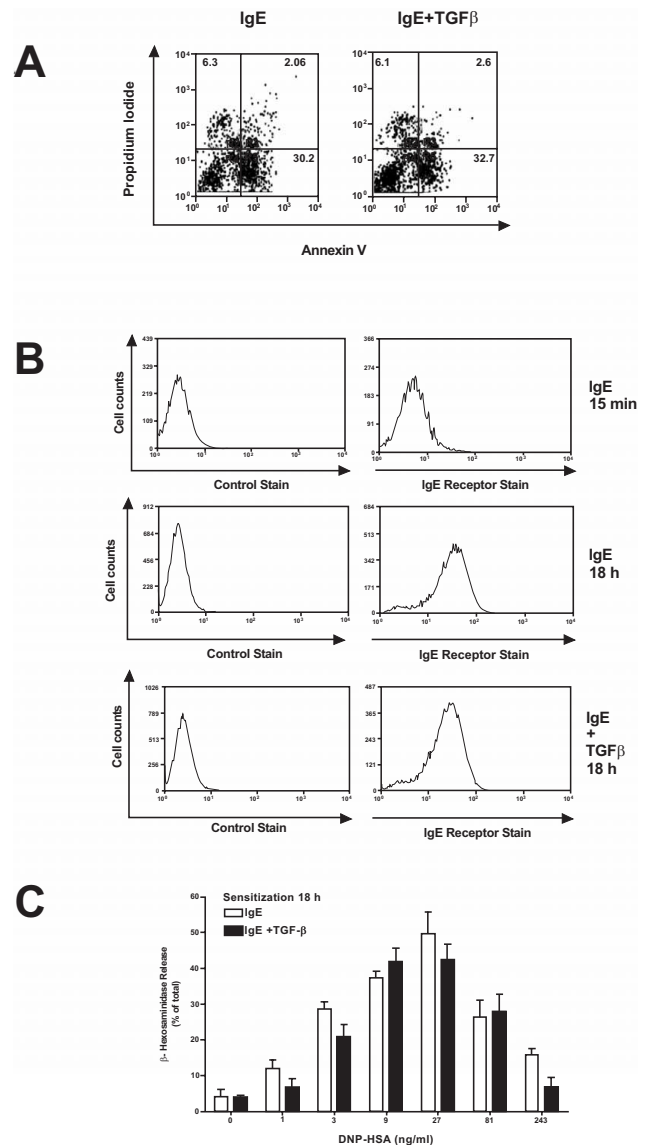
propidium iodide permeability. Fig. (2A) shows a representative dot plot of BMMCs after 18 h of sensitization with IgE or IgE+TGF $\beta$ . Early (annexin V positive; propidium iodide negative), or late (annexin V positive; propidium iodide positive) apoptotic cells were found without change in cells sensitized with IgE or with IgE+TGF $\beta$  for 18 hours. Percentages of annexin V positive cells in IgE condition *versus* IgE+TGF $\beta$  were 25%  $\pm$  10% *vs.* 32%  $\pm$  14% (mean values  $\pm$  SEM, n=3), and PI positive cells in IgE condition *versus* IgE+TGF $\beta$  were 6%  $\pm$  4% *vs.* 5%  $\pm$  2%; double positive (late apoptotic) cells did not show any change in the presence of TGF $\beta$ , since there were 2.5  $\pm$  0.6% of the total population, an usual value obtained from this type of primary culture [27]. Total cell numbers were found without change after the 18 hours-long incubation with IgE or IgE+TGF $\beta$ , suggesting no important effect on cell proliferation (data not shown). In order to explore if sensitization of mast cells with IgE+TGF $\beta$  could induce changes on the amount of Fc $\epsilon$ RI receptor at the plasma membrane, we analyzed Fc $\epsilon$ RI receptor expression using flow cytometry. As can be observed in Fig. (2B), the 18 hours-long sensitization period with IgE alone was able to induce the well-documented increase of Fc $\epsilon$ RI in the plasma membrane of mast cells [3], and this increase was not modified by the presence of 100 pM TGF $\beta$ . In order to test if sensitization with IgE+TGF $\beta$  could change the capacity of Fc $\epsilon$ RI to trigger degranulation,  $\beta$  hexosaminidase release was measured after antigen addition in IgE or IgE+TGF $\beta$  sensitized cells. Fig. (2C) shows that degranulation triggered by antigen addition was not altered in cells sensitized in the presence of TGF $\beta$ .

### IgE-TGF $\beta$ -Sensitized Mast Cells Secrete VEGF after Fc $\epsilon$ RI Crosslinking Through a Transcription-Independent Mechanism

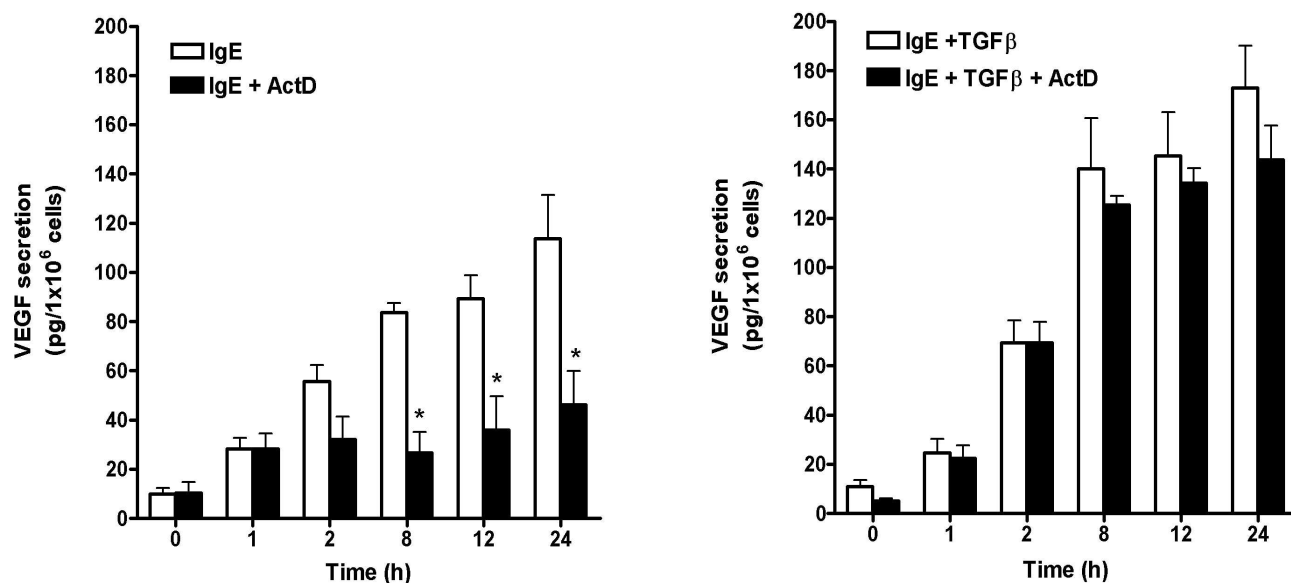
VEGF production in different cell types involves *de novo* transcription, mRNA stabilization and/or differential processing of the protein [28]. In order to analyze the mechanism by which VEGF production was increased after antigenic stimulation of IgE+TGF $\beta$  sensitized cells, we tested the effect of the transcription inhibitor Actinomycin D (ActD) on Fc $\epsilon$ RI-induced VEGF secretion. Fig. (3) shows VEGF secretion in IgE-(left panel) and IgE+TGF $\beta$ -sensitized cells (right panel) after Fc $\epsilon$ RI crosslinking in the presence/absence of ActD (5 $\mu$ g/mL). Cells were sensitized for 18 hours, washed once, and incubated with the inhibitor 15 min before antigen addition. VEGF secretion was evaluated at different times after stimulation. It was possible to observe that Fc $\epsilon$ RI crosslinking induces VEGF secretion through a mechanism sensitive to ActD on IgE-sensitized cells, whereas the processes involved on VEGF production in IgE+TGF $\beta$  sensitized cells was insensitive to the transcription inhibitor. Cell viability (determined by trypan blue exclusion) was not altered by the longest time of incubation with ActD (data not shown).

### Sensitization of Mast Cells in the Presence of TGF $\beta$ Causes a Marked Increase on VEGF mRNA Half-Life after IgE-Antigen Stimulation

Since VEGF secretion in IgE+TGF $\beta$  sensitized cells was not prevented by ActD pre-treatment, we decided to evaluate VEGF mRNA turnover after Fc $\epsilon$ RI stimulation using primers able to amplify the three main isoforms of VEGF



**Fig. (2).** TGF $\beta$  presence during IgE-dependent sensitization of mast cells does not induce apoptosis neither modify Fc $\epsilon$ RI receptor expression or antigen-induced degranulation. Panel A. BMMCs were incubated 18h with IgE or with IgE +TGF $\beta$ . After treatment, cells positive to propidium iodide, annexin V or both, were analyzed by flow cytometry. Numbers inside the graphs show the percentage of cells in each region of a representative experiment (of three performed). Panel B. BMMCs were incubated with IgE during 15 minutes or 18 h, or with IgE+TGF $\beta$  for 18 hr. Fc $\epsilon$ RI expression was assessed by flow cytometry using a FITC-labeled isotype control antibody (left histograms) or a specific FITC-coupled anti-IgE antibody (right histograms). A representative result from three different experiments using distinct BMMC cultures is shown in each case. Panel C. BMMCs were sensitized with IgE or IgE+TGF $\beta$  during 18 h. After this time, cells were stimulated with different concentrations of DNP-HSA and  $\beta$  hexosaminidase release was determined as described in the Material and Methods section. Data are expressed as the means  $\pm$  SEM from three different experiments using distinct cell cultures.



**Fig. (3).** BMMC sensitization with IgE+TGF $\beta$  causes antigen-induced secretion of VEGF in a transcription-independent fashion. BMMCs were sensitized 18 h with IgE (left panel) or IgE+TGF $\beta$  (right panel) and after that, cells were washed once and suspended in fresh media containing (or not) 5  $\mu$ g/mL Actinomycin D (ActD) for 15 minutes. Then, antigen (27 ng/mL) was added and supernatants were taken at different times of incubation at 37°C. VEGF concentration was determined using specific ELISA kits. Data are expressed as the means  $\pm$  SEM from three different experiments using distinct cell cultures. \*  $p < 0.05$ , with respect to IgE-sensitized cells.

expressed in mice (VEGF<sub>120</sub>, VEGF<sub>164</sub> and VEGF<sub>188</sub>). Fig. (4) shows that VEGF mRNA is abundant in BMMCs and its half-life is short ( $\approx$  25 min) in non-sensitized/non stimulated cells, indicating a continuous synthesis and degradation of VEGF mRNA. IgE and IgE+TGF $\beta$ -sensitized cells were treated with antigen in order to activate Fc $\epsilon$ RI signaling system and, 15 minutes later, ActD was added in order to stop *de novo* transcription. Total RNA was isolated at different times after ActD addition and remaining VEGF mRNA was detected by RT-PCR. In those conditions, the amount of VEGF mRNA induced by 15 min of antigen stimulation in IgE sensitized mast cells was not different from the basal observed in non-sensitized, non-stimulated mast cells (lane 1 vs lane 5 on Fig. 4A). However, it was possible to observe a marked stabilization of VEGF mRNA induced by antigen stimulation in IgE-sensitized mast cells (VEGF mRNA half-life  $> 100$  min). Interestingly, in cells sensitized with IgE+TGF $\beta$ , VEGF mRNA half-life was increased to more than 3 h after Fc $\epsilon$ RI stimulation. This result suggests that presence of TGF $\beta$  during the IgE-dependent sensitization of mast cells induce changes on the Fc $\epsilon$ RI signal transduction system allowing to an increased VEGF mRNA stabilization after antigenic stimulation.

#### VEGF Secretion and mRNA Stabilization after Fc $\epsilon$ RI Crosslinking Depends on p38 MAPK Activity

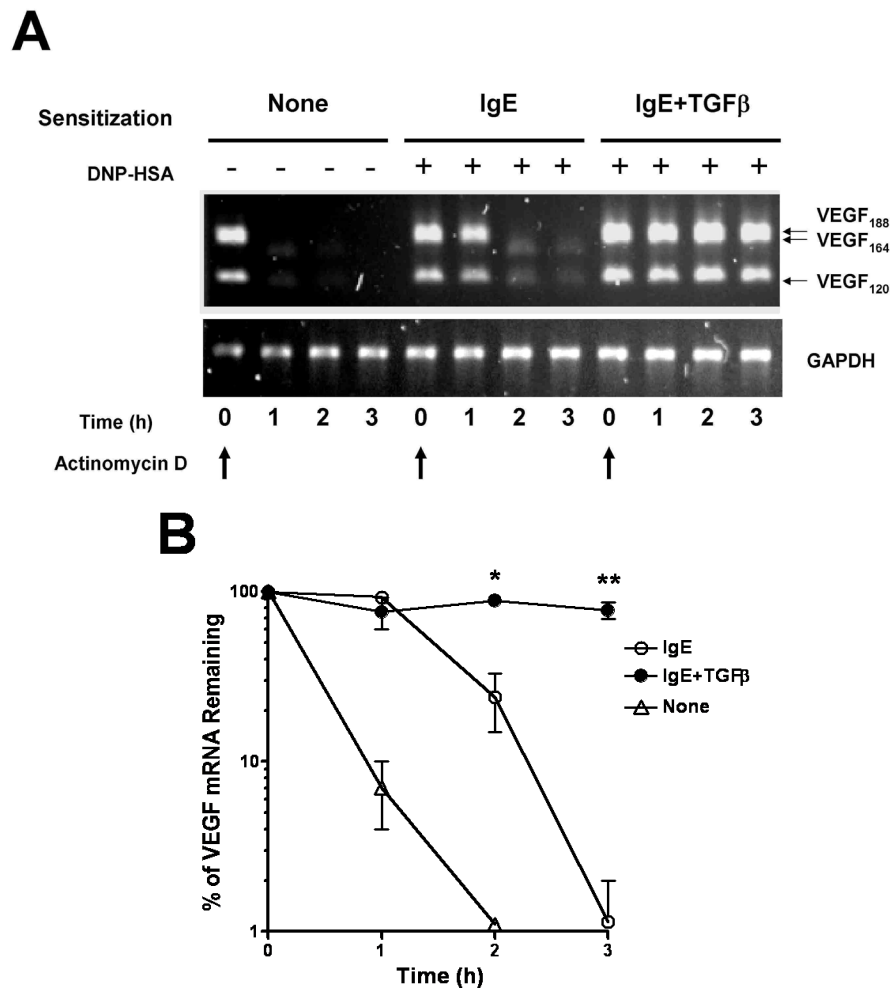
It has been reported that activation of different MAPK, in particular p38, controls VEGF mRNA half life [29]. In order to characterize the pathway responsible for VEGF mRNA stabilization in IgE-antigen activated mast cells, we decided to explore the participation of p38 MAPK using the selective inhibitor SB202190 [30]. IgE or IgE+TGF $\beta$  sensitized mast cells were treated with different concentrations SB202190 for 15 minutes. Then, antigen was added and VEGF secretion was measured in cell supernatants after 8 h (Fig. 5A). Incubation with SB202190 previous to antigen stimulation

was able to block Fc $\epsilon$ RI-induced VEGF secretion in both IgE and IgE+TGF $\beta$  sensitized cells and the inhibition was evident at micromolar concentrations of the compound. Differences on sensitivity to SB202190 were noticed between IgE and IgE+TGF $\beta$  sensitized cells; a detailed analysis of the inhibition was performed and is presented in the inset of Fig. (5A), where it can be observed that the slope of the straight line obtained with the inhibition data was -0.65 for IgE-sensitized cells and -0.96 for IgE+TGF $\beta$ . Those results indicate that Fc $\epsilon$ RI-induced VEGF secretion in IgE+TGF $\beta$  sensitized cells is more sensitive to SB202190 than the observed in IgE alone-sensitized BMMCs. This increased sensitivity was evident at 0.1  $\mu$ M SB202190, where the difference between inhibitor-free and inhibitor-treated IgE+TGF $\beta$  sensitized cells showed statistical significance, whereas that was not the case for inhibitor free and inhibitor-treated IgE-sensitized cells.

In a second group of experiments, IgE and IgE+TGF $\beta$  sensitized cells were treated with 10  $\mu$ M SB202190 for 15 minutes before the addition of antigen. Antigen was added and 15 min later, ActD was added in order to stop *de novo* gene transcription. Cell samples were taken at different times after ActD addition and total RNA was purified in order to detect the amount of VEGF mRNA by RT-PCR. As can be observed in Fig. (5B), pretreatment with SB202190 did not change the amount of basal or 15 min antigen-induced VEGF mRNA accumulation, however, it was able to prevent the observed stabilization of VEGF mRNA in both IgE and IgE+TGF $\beta$  sensitized cells after antigen stimulation.

#### DISCUSSION

Diverse and sometimes contradictory effects of TGF $\beta$  on immune cells have been reported. The role of TGF $\beta$  as a suppressor of immunity and inflammation is supported by a number of studies showing the inhibitory effects of TGF $\beta$  on



**Fig. (4).** TGF $\beta$  presence during IgE-dependent sensitization of mast cells causes an increase on VEGF mRNA half-life after Fc $\epsilon$ RI stimulation. Panel **A**. BMMCs growing in complete media were treated 18 h with none, IgE or IgE+TGF $\beta$ . After this time, cells were washed once and suspended in new media containing 27 ng/ml DNP-HSA for 15 minutes before the addition of 5  $\mu$ g/mL ActD. Cell samples were taken at different times after ActD addition and RT-PCR was performed as described in the Material and Methods section. Results of a representative experiment (from three performed with different cultures) are shown. Panel **B**. Densitometric analysis of the bands obtained in panel **A** was performed and results were used to construct a graph showing VEGF mRNA decay. VEGF signal was normalized to its respective GAPDH intensity value. Values at t=0 after ActD addition on panel **A** were taken as 100%. Data are expressed as the means  $\pm$  SEM from three different experiments using distinct cell cultures. \*  $p < 0.05$  and \*\*  $p < 0.01$ , with respect to IgE-sensitized cells.

immune cell function, including mast cells [8]. Long-term mast cell culture in the presence of TGF $\beta$  provoked low expression of Fc $\epsilon$ RI in the plasma membrane and impaired IgE-mediated cytokine secretion after antigenic stimulation [31]; TGF $\beta$  was also able to inhibit IL-3 and IL-4 dependent mouse connective tissue mast cell proliferation [32]; also, TGF $\beta$ -induced alterations in proliferation of BMMC cultures without any change in mast cell function have been reported [33]. On the other hand, an increase on some of the IgE-antigen-elicited responses in mast cells has been documented. Shorter (16 h) periods of mast cell exposure to TGF $\beta$  have been shown to promote an increased lymphotactin mRNA production after Fc $\epsilon$ RI crosslinking [34]. The increase of Fc $\epsilon$ RI-induced synthesis of lipid mediators, such as leukotriene (LT)A $_4$ , LTB $_4$ , LTC $_4$  and 6-trans LTB $_4$  after TGF $\beta$  treatment, without any modification on  $\beta$ -hexosaminidase release has been observed [35]. Different circumstances can explain the contradictory effects of TGF $\beta$  on Fc $\epsilon$ RI-induced responses in mast cells. Distinct times and

concentrations of TGF $\beta$  have been used in each study; distinct amounts of IgE and diverse protocols of sensitization have been used too. It is possible that the final effects of TGF $\beta$  depend on the utilized doses and the presence/absence of IgE during long periods of time. In our experiments, a positive influence of TGF $\beta$  on Fc $\epsilon$ RI-induced VEGF secretion was observed when saturating amounts of IgE were used, at the same time, to sensitize mast cells. The observed effect was not related to apparent changes on mast cell apoptotic rate or IgE-receptor expression in plasma membrane (Fig. 2).

Different molecular mechanisms can contribute to the increase in VEGF secretion after Fc $\epsilon$ RI triggering and some of them could be modified by the presence of TGF $\beta$  during the sensitization phase of mast cells. As can be observed in Fig. (3), VEGF secretion after Fc $\epsilon$ RI crosslinking strongly depends on *de novo* transcription in IgE-sensitized cells, whereas in IgE+TGF $\beta$ -sensitized cells the contribution of

transcription seems to be discrete. These results suggest that TGF $\beta$  presence during the sensitization phase of mast cells causes a remodeling of the pathway that controls VEGF secretion after Fc $\epsilon$ RI crosslinking, altering the mechanisms responsible for VEGF mRNA stability or protein synthesis/secretion. Studies about the molecular events leading to VEGF production after Fc $\epsilon$ RI receptor crosslinking in mast cells are scarce, but some information can be taken from experiments done in hypoxic conditions in other cell types. Induction of VEGF during hypoxia depends on the activation of HIF transcription factor, which is able to increase VEGF mRNA amounts 2-3 times [28]. However, VEGF production also depends on the activation of several post-transcriptional mechanisms leading to VEGF mRNA stabilization in the cytoplasm, producing an increase in VEGF mRNA half life by a factor of 3, resulting in 8-30 times higher levels of VEGF mRNA [36].

Since VEGF is tightly regulated at post-transcriptional level, we decided to explore VEGF mRNA turnover after antigenic stimulation in cells sensitized with IgE or IgE+TGF $\beta$  and, as a control, we determined VEGF mRNA half-life in non-sensitized/non stimulated cells too. Notably, the amount of VEGF mRNA induced by 15 min of antigen IgE-sensitized mast cells was not different from the basal observed in non-sensitized, non-stimulated conditions (lane 1 vs lane 5 on Fig. 4A), indicating that sensitization with monomeric IgE does not modify basal VEGF mRNA accumulation and that transcriptional activation of VEGF is not evident at short times (15 min) after antigenic stimulation. The main effect of the presence of antigen in IgE-sensitized cells seemed to be the increased half-life of VEGF mRNA, suggesting that binding of monomeric IgE to its receptor during the sensitization step could induce some changes on the mast cell apparatus controlling inflammatory mRNA accumulation after antigenic stimulation, leaving the cells able to inhibit normal rates of mRNA degradation or allowing the production of proteins able to stabilize distinct mRNA molecules. IgE binding once was thought to be a passive step which would keep mast cells in a "prepared" state before receptor aggregation with multivalent allergens, but this view of the sensitization process has been challenged after the findings showing that receptor-IgE interaction can promote several changes on mast cell activity, such as up regulation of cell surface expression of IgE receptor, selective cytokine production and survival of mast cells [37]. Our results provide evidence suggesting those changes could include the synthesis of post-transcriptional regulators of cytokine synthesis.

Interestingly, the presence of TGF $\beta$  during the sensitization step significantly improved VEGF mRNA stabilization after antigenic stimulation. TGF $\beta$ -induced stabilization of COX-2, thrombospondin, parathyroid hormone, ribonucleotide reductase R2 subunit, amyloid precursor protein, and the hyaluronan receptor mRNAs by activation of Smad-dependent and independent pathways has been reported [38]. Since no change on basal levels of VEGF mRNA was observed in cells sensitized with IgE+TGF $\beta$  (Fig. 4), a direct effect of TGF $\beta$  on basal mRNA stabilization cannot be claimed, but it is possible that during the period of sensitization, TGF $\beta$  could synergize with monomeric IgE for the production of specific proteins leading to mRNA stabilization after Fc $\epsilon$ RI crosslinking. Distinct proteins have been

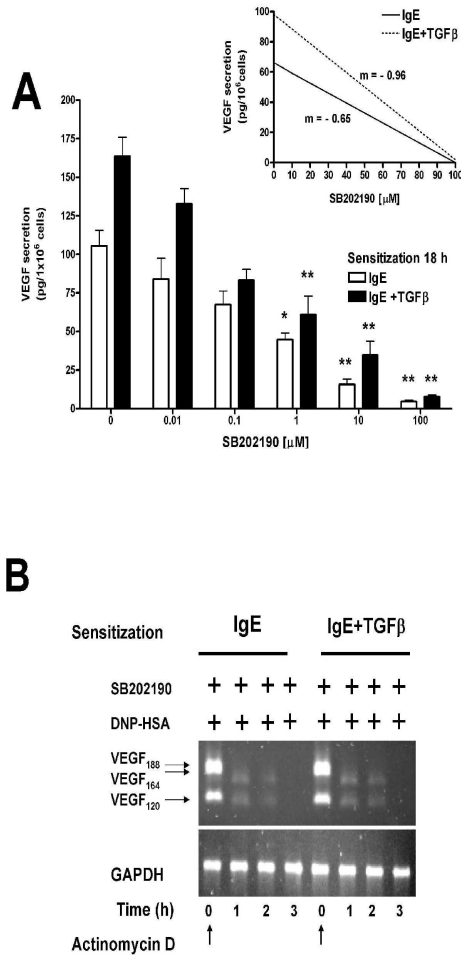
involved on VEGF mRNA stability control. HuR (a member of the embryonic lethal abnormal vision, ELAV, family) [39], tristetrapolin (TTP), and other factors [40] are able to bind and control VEGF mRNA half-life in different conditions. Changes on their synthesis, activation or subcellular localization due to TGF $\beta$  presence could explain the changes observed on VEGF mRNA.

VEGF mRNA half-life was increased more than three times after antigen stimulation in IgE+TGF $\beta$ -sensitized cells (Fig. 4). However, the increase on VEGF secretion was not of the same magnitude (50% increase respect to IgE-alone sensitized cells, Fig. 1). These results suggest other post-transcriptional processes, such as translational efficiency or selective secretion can contribute to VEGF production in mast cells. It has been reported that, although 3'ARE containing mRNAs are highly stabilized by HuR binding in the cytoplasm, those messengers do not co-localize with the polysomal fraction in cell extracts and its translation is not proportionally increased [41]. Although VEGF has been found inside histamine-containing granules in mast cells [23] slower mechanisms of secretion, like piecemeal degranulation [42] could participate in VEGF production in mast cells and could be modified during the sensitization process. Future experiments analyzing the intracellular VEGF content in sensitized mast cells, its secretion rate and the effects on the formation of new blood vessels in allergic inflammation, will give light on the mechanism and the biological consequences of the observed effects of TGF $\beta$ .

p38 MAPK has been reported to be important for VEGF, TNF- $\alpha$  and IL-1 $\beta$  mRNA stabilization [43]. Pretreatment of BMMCs with SB202190 was able to block the Fc $\epsilon$ RI-dependent phosphorylation of the ATF-2 transcription factor, a well recognized downstream effector of p38 pathway (data not shown). As can be observed in Fig. (5), SB202190 pretreatment did not modify the amount of VEGF mRNA after 15 minutes of antigenic stimulation, but it was able to block the previously observed antigen-induced mRNA stabilization (Fig. 5), indicating the participation of p38 MAPK on the pathway connecting Fc $\epsilon$ RI receptor with VEGF mRNA turnover. Two important substrates of p38 $\alpha$  are the MAP kinase-activated protein kinase 2 (MAKAP2 or MK2) and the closely related family member (MK3) [44]. When these substrates are phosphorylated, they have been shown to activate various proteins and, particularly, MK2 has been found to phosphorylate TTP, altering TNF- $\alpha$  mRNA half-life [45]. Interestingly, VEGF secretion in IgE+TGF $\beta$ -sensitized cells seemed to be more sensitive to the effects of SB202190 than the observed in IgE alone-sensitized cells (Inset Fig. 5), suggesting an important effect of TGF $\beta$  in the induction of p38-dependent pathways leading to mRNA stabilization. Accordingly with the positive role of p38 on increased inflammatory cytokine release, IgE+ TGF $\beta$ -sensitized cells showed higher TNF- $\alpha$  secretion than IgE-sensitized cells after antigen stimulation, and that increase was blocked by SB202190 (unpublished results).

An important role of TGF $\beta$  in allergic inflammation has been particularly observed in chronic asthma. The chronic inflammatory response in the airway is characterized by the presence of increased numbers of Th2 lymphocytes, eosinophils and activated mast cells. The airways of patients with asthma exhibit varying levels of structural changes (airway





**Fig. (5).** p38 MAPK activity is necessary for Fc $\epsilon$ RI-dependent VEGF secretion and mRNA stabilization in both IgE and IgE+TGF $\beta$  sensitized mast cells. Panel **A**. BMMCs were sensitized with IgE or IgE+TGF $\beta$  for 18 hours. Cells were then washed once and suspended in fresh media containing vehicle or different concentrations of SB202190. After 15min incubation at 37°C, DNP-HSA (27 ng/ml) was added and cells were incubated for 8 hours at 37°C prior to supernatant collection. VEGF concentration in the supernatants was determined by ELISA. Data are expressed as the means  $\pm$  SEM from three different experiments using distinct cell cultures. \*\*\* p<0.05 with respect to IgE sensitized cells, paired t-test, & p<0.05 with respect to IgE+TGF $\beta$ -sensitized, inhibitor free cells; # p<0.05 with respect to IgE-sensitized, inhibitor free cells, one way ANOVA. Inset. Linear least squares regression performed using values from the shown bar graph. Equation for IgE-sensitized cells was  $y = -0.657(x) + 65.79$ ; for IgE+TGF $\beta$ -sensitized cells, equation was  $y = -0.963(x) + 98.17$ . Panel **B**. BMMCs were sensitized 18 h with IgE or IgE+TGF $\beta$ . Then, cells were washed once and suspended in new media containing vehicle or 10  $\mu$ M SB202190 for 15 min at 37°C. Cells were then stimulated for 15 min with 27 ng/mL DNP-HSA before 5  $\mu$ g/mL ActD addition (t=0). After ActD addition, cell samples were taken at different times and RT-PCR was performed as described in the Material and Methods section. Results of a representative experiment (of three performed in with different cultures) are shown.

remodeling) that modify lung function. Characteristic structural changes of airway remodeling include epithelial cell mucus metaplasia, smooth muscle hypertrophy/hyperplasia,

eosinophilia, subepithelial fibrosis and increased angiogenesis [46]. Recent studies suggest that eosinophil-derived TGF $\beta$  may play an important role in the pathogenesis of airway remodeling [47]. On the other hand, endobronchial biopsies from patients with asthma showed increased numbers of chymase-positive mast cells associated with both increased numbers of blood vessels and increased numbers of cells expressing VEGF compared with healthy control subjects [48]. Co-localization immunohistochemistry studies demonstrated that mast cells are an important source of VEGF [49]. Pulmonary mast cells are sensitized binding allergen-specific IgEs and the release of histamine and inflammatory mediators is triggered by allergen-induced Fc $\epsilon$ RI crosslinking [2]. Our results suggest that TGF $\beta$  presence in lung could modify the IgE-dependent sensitization process, leading to an increase of VEGF secretion after allergen contact. Previous evidence has implied TGF $\beta$  in extracellular matrix deposition in asthma [50], our results indicate that TGF $\beta$  could participate in lung angiogenesis due to its effects on VEGF secretion by mast cells. Interestingly, using an OVA-sensitized rat model of allergic lung inflammation, it has been shown that an inhibitor of TGF $\beta$  receptor type I kinase, has both a preventive and neutralizing effect on airway inflammation, as measured by basic-protein positive eosinophils and CD2+ T-cell counts in mucosal airways, cell proliferation and goblet cell hyperplasia induced by repeated allergen challenges [51], supporting the role of a pro-inflammatory role of TGF $\beta$  in mast cell-dependent inflammatory reactions.

Mast cells are in contact with soluble and matrix-associated TGF $\beta$  during different circumstances, since they finish their differentiation in the presence of microenvironment components in distinct tissues [1]. Mast cells interact to basement membrane during migration and maturation in peripheral tissues, such as the gastrointestinal and genitourinary tracts and the respiratory system. In the road to different locations, TGF $\beta$  induce phenotypic changes, preparing mast cells for posterior increased IgE-antigen-elicited responses [52]. Evidence of increase of mast cell hyperplasia in connection with TGF $\beta$  presence has been found in disorders such as rheumatoid arthritis, pulmonary fibrosis, and cancer, suggesting an important influence of TGF $\beta$  on mast cell maturation, proliferation and signaling [53]. This modulator effect of TGF $\beta$  on Fc $\epsilon$ RI-dependent responses could be exerted also by other local mediators like adenosine, acetylcholine or neuropeptides, present in lung or close to nerve terminals; i.e., a positive effect of thrombin on some IgE/antigen-elicited responses in mast cells has been reported [54].

Our *in vitro* results suggest that a potentially important synergism between pro-inflammatory actions of TGF $\beta$  and monomeric IgE can occur in different inflammation-related pathologies. Future studies analyzing the molecular remodeling of the Fc $\epsilon$ RI receptor signal transduction system, due the presence of locally produced mediators together with allergen-specific IgEs, will improve our knowledge on the specific allergic responses observed in distinct tissues due to particular microenvironment conditions.

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#### LIST OF ABBREVIATIONS

|                  |   |                                    |
|------------------|---|------------------------------------|
| BMMC             | = | Bone Marrow-derived Mast Cells     |
| VEGF             | = | Vascular Endothelial Growth Factor |
| IgE              | = | Immunoglobulin E                   |
| Fc $\epsilon$ RI | = | High Affinity IgE Receptor         |
| TGF $\beta$      | = | Transforming Growth Factor $\beta$ |
| VEGFR2           | = | VEGF Receptor 2                    |
| ARE              | = | AU-rich cis-element                |

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