Original Article

Effect of Transforming Growth Factor-β (TGF-β) on proliferation of gastric epithelial cells in culture

M.Hossein Ghaini, MD[1] T. Ghazanfari, PhD[2] N. Fallah, PhD[3]

1.Dept. pathology, Medical Faculty of Shahed University, Tehran, Iran
2. Dept. Immunology, Medical Faculty of Shahed University, Tehran, Iran
3. Dept. Social Medicine, Medical Faculty of Shahed University, Tehran, Iran

ABSTRACT

Objective: Helicobacter pylori has a well-established role in the development of gastric cancer. In vitro studies reveal increased proliferation of the gastric mucosa in the presence of H. pylori infection. It has been also shown that production of some cytokines, such as interleukin-1 beta (IL-1b) is increased in H. pylori infection. In addition, IL-1b increases proliferation of gastric epithelial cell in culture study. In this study, The effect of transforming growth factor beta (TGF-b) on gastric epithelial cell proliferation has been examined.

Materials and methods: AGS cells were cultured with TGF-b. DNA synthesis was evaluated by bromo-deoxyuridine (BrdU) test and total viable cell numbers by MTT assay.

Results: TGF-b decreased DNA synthesis and cell numbers. This effect was both time- and dose-dependent ($p<0.05$). Both tests, BrdU test & MTT assay revealed this suppressive effect, but it was more evident in BrdU test.

Conclusion: TGF-b suppresses proliferation of malignant gastric epithelial cells. It appears that modulatin of tyrosine kinase activity is essential to anti-proliferative effect of TGF-b. Decreased gastric epithelial proliferation due to TGF-β may contribute to the decreased risk of gastric cancer and precancerous lesions in *H. pylori*-infected individuals or slowing the progression of disease in gastric cancer patients.

Key words:TGF-β, gastric epithelial cells

Introduction

Helicobacter pylori is believed to be the major etiological factor in the development of gastric adenocarcinoma. Large-scale epidemiological studies have confirmed a strong association between *H. pylori* infection and both cancer and the earlier histological stages, atrophy and intestinal metaplasia (1); both of which increase the risk of later neoplastic transformation. Animal models have also demonstrated the importance of

Received: 20 Novemer 2005
Accepted: 28 December 2005
**Address communications to: M. H. Ghaini Dept. pathology,mostafa khomeini hospital-Tehran-Iran*
E-mail: Ghaini46@yahoo.com

H. pylori in gastric carcinogenesis. Increased rates of proliferation of the gastric mucosa are typical in *H. pylori* infection, and hyperproliferation within the gastrointestinal tract appears to be a marker for later malignant change. The cause of the increased rate of proliferation is not clear, but the increased rates reduce to normal with clearance of the infection. Although hypeprproliferation is typical *in vivo,* studies testing the effects of *H. pylori* or its products *in vitro* have shown conflicting results, with both enhanced and diminished proliferation reported (2,3). It is possible that other components of the inflammatory response typical of *H. pylori* infected mucosa could be at least partly responsible for driving the increased cell proliferation.

There has been an increasing interest to determine the role of individual cytokines in the process of gastric mucosal injury during recent years; for example the pluripotent pro-inflammatory cytokine interleukin-1β (IL-۱β) has a central role in the pathogenesis of *H. pylori*-induced mucosal inflammation (4) . IL-1β gene expression and protein production are increased in *H. pylori* infection and reduce with successful eradication. The presence of the IL-1β genotype polymorphism associated with enhanced IL-1β-production has been associated with a significant increased risk of gastric cancer and pre-cancerous lesions. Recognition of IL-1β role, leads to assessment of other cytokines, such as TGF-β, that generally antagonize most of IL-1β functions.

Present studies reveal controversial data about TGF-β. Although, it is generally believed that TGF-β is an antiproliferative agent, there are some studies that show the reverse effect; skin keratinocytes and endothelial cells are some examples of proliferation-inducing effect of TGF-β. In addition, there are confusing evidences for TGF-β in gastric cancer. TGF-β expression has been detected in about 70% of gastric tumors, more frequently in the intestinal-type. This expression is correlated with p21 expression, but not correlated with stage and prognosis. It has been shown that TGF-β expression in normal gastric mucosa of first-degree relatives of patients with gastric cancer, is more than others. Serum level of TGF-β is elevated in gastric cancer patients and has an inverse relation with prognosis. It is notable that in experimental studies, those gastric tumors that secret greater amounts of TGF-β, were more therapeutically resistant. There is no elevation of TGF-β synthesis by gastric epithelial cells in the presence of H. pylori, however.

There are three isoforms of TGF-ß: TGF-ß1, TGF-ß2, and TGF-ß3. Each isoform is encoded by a distinct gene and is expressed in both a tissue-specific and a developmentally regulated fashion(5). All three isoforms are highly conserved in mammals, suggesting a critical biologic function for each isoform. These isoforms differ in their binding affinity for TGF-ß receptors, and the deletion of individual isoforms in mice results in different phenotypes. Each TGF-ß isoform is synthesized as part of a large precursor molecule containing a propeptide region in addition to TGF-ß. TGF-ß is cleaved from the propeptide before the precursor is secreted by the cell but remains attached to the propeptide by noncovalent bonds. The attachment of TGF-ß to the binding protein by disulfide bonds prevents it from binding to its receptors (6). There are four latent TGF-ß–binding proteins; they are encoded by distinct genes and are expressed in a tissue-specific fashion. TGF-ß may be activated by plasmin-mediated cleavage of the complex (7). Since TGF-ß and its receptors are present in most cells, this activation is probably a critical regulatory step in the action of TGF-ß.

TGF-ß regulates cellular processes by binding to three high-affinity cell-surface receptors known as types I, II, and III. The type III receptors are the most abundant type. They function by binding TGF-ß and then transferring it to its signaling receptors, the type I and II receptors. The

nonsignaling role of type III receptors is shared by other abundant proteoglycan cytokine receptors, including syndecan for fibroblast growth factor, p75 for nerve growth factor, and the type II receptor for insulin-like growth factor.

Consider that most cases of gastric cancer, in contrast to the most of epithelial cancers, is not preceded by hypertrophy. Actually, mucosal atrophy and consequent hypochlorhydria induce malignant changes. Alteration of gastric proliferation by TGF-β might change the carcinogenic process. Therefore the direct effects of TGF-β on gastric epithelial proliferation have been assessed.

Material & Methods

I) Cell culture

The human AGS gastric carcinoma cell line was purchased from the Pasteur institute (Tehran, Iran). Cells were grown in monolayer culture in RPMI 1640 medium supplemented with 10 % fetal calf serum. Cells were grown in 50 cm^2 tissue culture flasks at 37°C in an atmosphere of 5% CO_2 and 95% air and passaged every 48 hours.

II) Proliferation studies

1-BromodeoxyUridine Test (BrdU).

Cells were grown in media containing 10% foetal calf serum, plated into 96-well plates at 10^5 cells/well and allowed to attach overnight. After washing with serum-free media, cells were incubated in serum free medium containing 10μL BrdU labeling solution for 4 hours in the presence of increasing concentrations of TGF-β. DNA synthesis was estimated by measurement of BrdU incorporation. Then, after removal of labeling medium, add 200 μL fixative and denaturing solution (FixDenat) to each well, and incubate for 30 min. at room temperature. After removal of solution, add 100μL peroxidase-containing antibody to each well. After 90 min. at room temperature, and three times washing, final step is performed by adding 100μL substrate to each well; and read the absorbance by ELISA reader at 450nm.

2-Cell growth

Total viable cell numbers were assessed by a modified MTT (3-[4,5-dimethylthiazol-2-yl]-2.5 diphenyl tetrazolim bromide assay) . Cells were plated into 96-well plates in medium containing 10% fetal calf serum. After attachment overnight, the medium was changed to 1% fetal calf serum-supplemented medium and increasing concentrations of IL-1β were added. Cells were cultured for 48 hours and then the medium was removed and fresh RPMI 1640 medium containing 0.5 ng/ml MTT was added. Cells were incubated at 37°C for 3 hours. The medium was then removed and 0.04 M HCl in isopropanol was added to extract the reduced formazan product. The resulting optical density at 550 nm was determined.

III) Chemicals and reagents

Recombinant human TGF-α were purchased from Roche. RPMI 1640 was from Gibco BRL (Paisley, UK) and all other reagents were from Roche.

IV) Statistics

Cytokine-stimulated results where compared with control unstimulated cells on the same 96-well plate. Data were compared by one-way analysis of variance and Student's t-test to determine statistical significance. Each experiment as performed in triplicate on 4–6 occasions. Results are expressed as mean ± standard deviation. Differences with P values of < 0.05 were considered significant.

Results

I) Effect of TGF-β on BrdU incorporation

TGF-β caused a dose-dependent decrease in DNA synthesis as measured by BrdU incorporation. As shown in figure 1, significant suppression was seen with 1–100 ng/ml TGF-

β. The maximal suppression of 25 ± 6 % below control was seen with 10 ng/ml. The higher dose of 100 mg/ml was slightly less effective in suppressing proliferation.

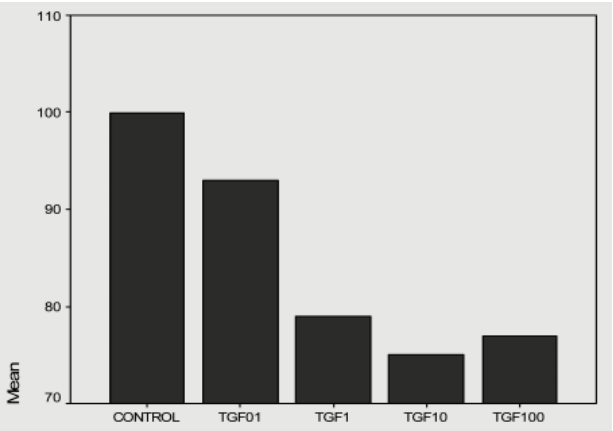

Fig1: Effect of TGF-β on AGS culture in BrdU Test

II) Effect of TGF-β on cell number

The decrease in DNA synthesis by TGF-β was translated into an absolute decrease in viable cell numbers. As shown in figure 2, TGF-β decreased cell numbers in a dose-dependent manner similar to the effects on BrdU test. The maximal suppression was again seen at 10 ng/ml of TGF-β, which produced a 22 ± 4 % decrease in total cell number.

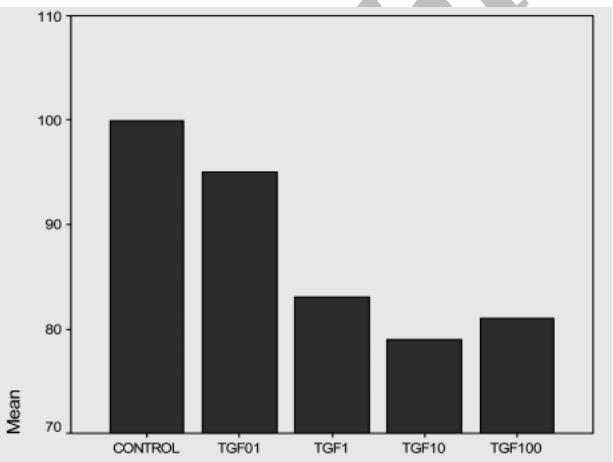

Fig 2: Effect of TGF-β on AGS culture in MTT Test

Discussion

This study demonstrated that TGF-β decreased proliferation of AGS cells. TGF-β supressed both BrdU incorporation, as a measure of stimulation of DNA synthetic rate and also total cell numbers as measured by the MTT assay. This illustrates that the supression of DNA synthesis by TGF-β is translated into a real increase in cell numbers.

The results of the current study suggest that TGF-β can directly suppress gastric epithelial proliferation. TGF-ß regulates cellular proliferation in a cell-specific manner (8). In most epithelial, endothelial, and hematopoietic cells, TGF-ß is a potent inhibitor of cell proliferation. It arrests the cell cycle in the G1 phase by stimulating production of the cyclin-dependent protein kinase inhibitor p15 and by inhibiting the function or production of essential cell-cycle regulators, especially the cyclin-dependent protein kinases 2 and 4 and cyclins A and E. Also, TGF-β with p53 and p73 gene products activate CDK inhibitors, such as p16 (INK4a). In consequence, retinoblastoma gene product (pRb), that is phosphorylated by CDK, remains hypophosphorylated and inactive. Decreased phosphorylation of the retinoblastoma gene product, Rb, allows it to bind to and sequester members of the E2F family of transcription factors. Sequestered E2F is then unable to stimulate the expression of genes that regulate progression through the cell cycle, such as c-*myc* and b-*myb*. As a result, tumoral cells arrest in G1 phase and could not pass to S phase. In cancer cells, mutations in the TGF-ß pathway have been described that confer resistance to growth inhibition by TGF-ß, thus allowing uncontrolled proliferation of the cells. Decreased gastric epithelial proliferation due to TGF-β may contribute to the subsideded risk of gastric cancer and precancerous lesions in *H. pylori*-infected individuals or slowing the progression of disease in gastric cancer patients.

There are conflicting data available concerning the direct effects of TGF-β on gastric epithelial proliferation (9). Although the current study and the study on the other gastric cell line, SNU-620 showed decreased proliferation , TGF-β increased proliferation of gastric cell line SNU-۲۱۶. The exact reasons for these discrepancies are not

clear; but they may reflect intrinsic differences between the cell lines, differences in activation and involvement of paracrine growth-promoting pathways, and especially aberrant signaling due to mutations of TGF-β receptors (۹). It is also possible that the multiple signalling pathways activated by TGF-β have differing effects on proliferation, the dominant effect depending on the complex inter-relationships of stimuli and signalling pathways under different circumstances.

In normal cells, TGF-ß acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation or apoptosis. In the initial stages of tumorigenesis, a cell loses its TGF-ß–mediated growth inhibition as a result of mutation or loss of expression of the genes for one or more components of the TGF-ß signaling pathway. About 15 percent of gastric cancers, 100 percent of pancreatic cancers and 80 percent of colon cancers have a mutation affecting at least one component of the TGF-ß pathway(10).

Mutations in the TGF-ß signaling pathway were initially demonstrated in the type II receptor, when several cancers were found to have mutations in or loss of expression of this receptor (11). The coding region of the gene for this receptor has a sequence of 10 consecutive adenine nucleotides; the addition or deletion of an adenine within this region results in the production of a truncated, functionally inactive receptor. Such mutations are found in colon, gastric, endometrial, and other cancers (12). The same mutations have been found in the tumors of patients with hereditary nonpolyposis colon cancer, a genetic disorder due to a germ-line mutation of DNA mismatch-repair genes.

TGF-β has also an apoptotic effect, which is an established action of this cytokine. Although TGF-beta1, is known to induce apoptosis, the molecular mechanism of this apoptosis is largely undefined. Cell cycle and TUNEL analysis show that, upon TGF-beta1 treatment, cells are initially arrested at the G1 phase and then driven into apoptosis. Of note, caspase-3 is activated in accordance with TGF-beta1-induced G1 arrest. Activated caspase-3 is targeted to cleave p21(cip1), p27(kip1), and Rb, which play important roles in TGF-beta-induced G1 arrest, into inactive fragments. Subsequently, Cdk2 is activated due to the cleavage of p21 and p27. Inhibition of Cdk2 activity efficiently blocks TGF-beta1-induced apoptosis, whereas it did not prevent caspase-3 activation or the subsequent cleavage of target proteins. In contrast, the suppression of caspase-3 activity inhibited the cleavage of target proteins, the activation of Cdk2, and the induction of apoptosis. Taken together, it seems that activation of caspase-3 by TGF-beta1 may initiate the conversion from G1 cell cycle arrest to apoptosis via the cleavage of p21, p27 and Rb, which in turn causes Cdk2 activation and, most significantly, Cdk2 activation as a downstream effector of caspase is a critical step for the execution of TGF-beta1-induced apoptosis.

TGF-β suppress the promoter region of C-MYC gene, which leads to decrement of c-myc proto-oncogene. TGF-β also stimulates production of an especial transcription factor, named by TGF-β inducible early response gene 1 (TIEG1), which has both of apoptotic and anti-proliferative effects, simultaneously.

Further studies are necessary to clarify the exact mechanisms involved in TGF-β suppression of gastric epithelial proliferation. It is necessary to complete the current study by further assessments of other mediators, blockers and cell surface receptors.

Reference

1- Meining A, Morgner A, Miehlke S, et al. Atrophy-metaplasia-dysplasia-carcinoma sequence in the stomach: a reality or merely an hypothesis? Best Pract Res Clin Gastroenterol 2001,Dec;15(6):983-98

2- Holck S, Norgaard A, Bennedsen M, et al. Gastric mucosal cytokine responses in Helicobacter pylori-infected patients with gastritis

and peptic ulcers. Association with inflammatory parameters and bacteria load. FEMS Immunol Med Microbiol 2003 May 25;36(3):175-180

3- Yamaoka Y, Kodama T, Kita M, et al. Relation between cytokines and Helicobacter pylori in gastric cancer. Helicobacter 2001 Jun;6(2):116-24

4- Beales I. Effect of Interlukin-1β on proliferation of gastric epithelial cells in culture. *BMC Gastroenterology* 2002 2:7-11

5- Massague J. TGF-ß signal transduction. Annu Rev Biochem 1998 67:753-791

6- Wrana JL, Attisano L, Wieser R, et al. Mechanism of activation of the TGF-ß receptor. Nature 1994 370:341-347

7- Massague J. The transforming growth factor-ß family. Annu Rev Cell Biol 1990 6:597-641

8- Norgaard P, Hougaard S, Poulsen HS, Spang-Thomsen M.Transforming growth factor ß and cancer. Cancer Treat Rev 1995 21:367-403

9- Ju HR, Jung U, Sonn CH. Aberrant signaling of TGF-beta1 by the mutant Smad4 in gastric cancer cells. Cancer Lett 2003 196(2):197-206

10- Maehara Y, Kakeji Y, Kabashima A, et al. Role of transforming growth factor-ß 1 in invasion and metastasis in gastric carcinoma. J Clin Oncol 1999 17:607-614

11- Myeroff LL, Parsons R, Kim SJ, et al. A transforming growth factor ß receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res 1995 55:5545-5547

12- Villanueva A, Garcia C, Paules AB, et al. Disruption of the antiproliferative TGF-ß signaling pathways in human pancreatic cancer cells. Oncogene 1998 17:1969-1978