Infliximab in experimental alkali burns of the oesophagus in the rat

V. Yukselen[1], E. Vardar[2], O. Yukselen[3], N. Oruc[4], C. Yenisey[5], A. Karaoglu[1], O. Ozutemiz[4]

(1) Department of Gastroenterology and (5) Department of Biochemistry, Faculty of Medicine, Adnan Menderes University, Aydin, Turkey ; (2) Department of Pathology, S.S.K. Izmir Training Hospital, Izmir, Turkey ; (3) Department of Pathology, Nazilli State Hospital, Nazilli, Turkey ; (4) Department of Gastroenterology, Faculty of Medicine, Ege University, Izmir, Turkey.

Abstract

Backgrounds : **We aimed to investigate the efficacy of infliximab, a chimeric TNF-α antibody, in the prevention of fibrosis in an experimental alkaline burn of the oesophagus in the rat.**

Methods : **Thirty-two Wistar albino rats divided into four experimental groups. Caustic oesophageal burn was induced by applying 37.5% NaOH to the distal oesophagus. Infliximab was given at a dose of 5 mg/kg via the intraperitoneal route. Group A (sham) animals were uninjured, group B had untreated oesophageal burns, group C had oesophageal burns treated with a single dose of infliximab on the first day, and Group D had oesophageal burns treated with infliximab on the first and 14th days. Efficacy of the treatment was assessed on the 28th-day by measuring stenosis index of the oesophagus and histopathological damage score, and biochemically by determining tissue hydroxyproline content.**

Results : **There was no significant difference between the Group B and the infliximab treated Groups C and D in means of tissue hydroxyproline content and histopathological damage scores. Stenosis index was not significantly different between the Group B, Group C, and Group D.**

Conclusion : **Anti-TNF-α treatment with infliximab does not ameliorate the degree of fibrosis in alkali burns of the oesophagus in the rat. Further evaluation of inflammatory and immunological events leading to stricture in alkaline oesophageal burns may provide new perspectives for the treatment of alkaline oesophageal burns.** (Acta gastroenterol. belg., **2008**, 71, **21-26**).

Key words : caustic injury, oesophageal burn, fibrosis, infliximab, stricture, corrosive oesophagitis.

1. Introduction

Caustic oesophageal burns of the oesophagus constitute a great medical problem both in immediate and subsequent late complications. The most important late complication of the caustic oesophageal burn is stricture formation. Many therapeutic options have been suggested to prevent stricture development (1-3). However, in severe caustic burn of the oesophagus these modalities are usually ineffective. In the literature corticosteroids were used in the only prospective, controlled study in children with corrosive injury of the oesophagus (4). Unfortunately the results of this study were also negative. In this study the degree of fibrosis was related only to the severity of the corrosive burn and the use of corticosteroids revealed no benefit in this clinical setting (4).

The initial event in the alkali burns of the oesophagus is liquefaction necrosis involving venous thrombosis, tissue necrosis, and sloughing (5). Tumor necrosis factor-alpha (TNF-α) as a first identified member of a large family of cytokines has an important role in mediating acute and chronic inflammation in different diseases like rheumatoid arthritis and inflammatory bowel diseases (6). In rheumatoid arthritis, continuous exposure to TNF-α and other cytokines transforms the synovial fibroblasts into activated, proliferating cells with a destructive phenotype (7). Infliximab is a chimeric IgG1 antibody that effectively inhibits both soluble and membrane-bound TNF-α (8,9). Recently, some experimental studies using infliximab in inflammatory conditions of rats and pigs have been conducted (10-14). Despite increasing knowledge implicating the role of TNF-α in the inflammation, neither clinical nor experimental trials "in prevention of fibrosis in caustic injuries of the oesophagus" have been conducted. Based on these reports we aimed to investigate the effect of infliximab in preventing fibrosis in an experimental model of alkali burn of the oesophagus, which finally leads to oesophageal fibrosis and stricture, in the rat.

2. Methods

This was a randomized, controlled, experimental study. The protocol was approved by the Experimental Ethics Committee of Ege University School of Medicine and by Ege University School of Medicine, Department of Experimental Surgery, Izmir, Turkey. All rats were housed at 24 ± 1°C under controlled lighting (12 h light/12 h darkness), humidity, and human activity. Animals were acclimatized to these conditions for 10 days before the experiments. All study groups were kept in identical wire-bottomed cages to prevent coprophagy.

2.1. *Experimental model*

After the ethics committee approved the project, a preliminary study was performed to standardize the experimental model and operative technique. Corrosive oesophageal burn was induced by modifying the experimental model described previously (15). After overnight

Correspondence to : Dr. Vahit Yukselen, Department of Gastroenterology, Adnan Menderes University, Faculty of Medicine, 09100, Aydin, Turkey.
E-mail : yukselenvahit@yahoo.com

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fasting, rats were anesthetized with xylasine hydrochloride (15 mg/kg) and ketamine hydrochloride (100 mg/kg) intraperitoneally. Through a median laparotomy, a 1.5-cm segment of the abdominal oesophagus was isolated. The cardioesophageal junction was tied externally with 2/0 silk ligature to prevent the corrosive agent from leaking into the stomach. A 6-French feeding catheter 48 mm in length was placed in the upper part of the abdominal oesophagus via the mouth. Proximally just under the diaphragm, the oesophagus was tied externally with a 2/0 silk ligature to prevent aspiration of the corrosive agent into the respiratory system. One milliliter of 37.5% NaOH solution (pH = 12) was infused through the catheter for 90s, and then the solution was aspirated back. Subsequently, distilled water was used to irrigate the burned area segment for 60s. The proximal 2/0 silk suture was cut and the catheter was pulled back orally under negative pressure to prevent aspiration. Then the distal 2/0 silk suture was cut and the abdominal incision closed. After the procedure, chow pellets were withdrawn for 48 hours postoperatively and for that period rats were maintained on 5% dextrose and Ringer Lactate solution. Then, the rats were fed with standard food and water ad libitum in standard laboratory conditions.

2.2. *Study design*

Thirty-two Wistar albino rats weighing 200-240 g were divided randomly into four groups. Group A (n:8), the sham laparotomy group (negative control group), was instilled with only 0.9% saline after preparation of the distal oesophagus and treated with intraperitoneal injection of 1 mL 0.9% saline. In group B (n:8), the untreated group (positive control group), standard oesophageal burn was produced and treated with intraperitoneal injection of 1 mL 0.9% saline. In group C (n:8) standard oesophageal burn was induced. After the procedure completed rats were waited to completely recover from anesthesia. Infliximab was solved in distilled water then diluted with saline solution and was applied at a dose of 5 mg/kg intraperitoneally one hour after the rats completely recovered. In group D, rats (n:8) had oesophageal burns and treated with infliximab (5 mg/kg via intraperitoneal route) both at one hour after the rats completely recovered and on 14th days. All rats were sacrificed on the 28th day.

2.3. *Histopathological evaluation*

An experienced pathologists performed histopathological analyses in a blind manner. The oesophageal lumen was washed out with 2 mL of 0.9% saline and the abdominal oesophagus plus stomach were removed en block. The abdominal oesophageal segment was transversally sectioned into two equal parts. The proximal portion was fixed in 10% neutral formaldehyde and embedded in paraffin wax. Four slides, each 4 µm in thickness, were prepared and stained with hematoxyline-eosine and Van Giesson connective tissue dye. The thickness of the oesophageal wall and the lumen diameter were measured under a millimetric ocular microscope as demonstrated previously (16). Oesophageal stenosis index (17) was calculated according to formula stenosis index = (wall thickness (A1 + A2)/2) / (lumen diameter (B1 + B2)/2). Preparations were also examined for histopathological damage. Tissue damage was scored on a scale in three different categories ; collagen deposition in the submucosa, damage to the muscularis mucosa, and damage and collagen deposition in the tunica muscularis for a total score of 0-5 (18).

2.4. *Biochemical method*

The distal portion of the transected abdominal oesophagus was stored in -80°C until the assay. The hydroxyproline content of the tissues was determined according to the method that described previously (19). The tissue samples were homogenized in an ultrasonic sonicator at approximately 4°C for 30 s in Eppendorf tubes. Samples were hydrolyzed with 100 µL in 2 mol/L NaOH for 15 min at 121°C (one atmosphere pressure) in an autoclave. Hydroxyproline was converted to pyrole-2-carboxylic acid by oxidizing the sample with 900 µL of a 0.056 mol/L chloramine-T solution. After the oxidation step, 1 mL Erlich's reagent was added and vortexed, and the samples were placed on the water-bath at 65°C for 20 min. The optic densities of color samples were measured at 550 nm by using the UV 160 Shimadzu spectrophotometer. The assay was sensitive to determine 0.025 mM hydroxyproline in each sample. The hydroxyproline concentrations were calculated by standard curve using standard solutions of L-hydroxyproline. The hydroxyproline content was expressed as µg/mg wet tissue.

2.5. *Statistical analysis*

All statistical analysis was performed using SPSS statistical software for Windows (release 10.0, SPSS Inc. Chicago, IL, USA). A one-way analysis of variance (ANOVA) was performed to search any differences between the groups in terms of stenosis index and OH-proline levels. Depending on the homogeneity of the variances, Dunnett C post-hoc test (for nonhomogeneous variances) was used in pair comparison of the groups in terms of hydroxyprolin levels. Histological scores were compared between the groups using Kruskall-Wallis variance analysis and Mann-Whitney U-test for nonparametric data. Results were considered statistically significant at level $P < 0.05$.

3. Results

Thirty-one rats survived the entire study. One rat in Group C, which died before the end of the 28-day period, underwent necropsy, died of oesophageal perforation. Experimental data are presented in Table 1.

Table 1. — **Results of the hydroxyproline level, stenosis index, and histopathological damage score of the groups**

		Hydroxyproline level (μg/mg wet tissue)	Stenosis index	Histopathological damage score
Group	n	Mean ± SD	Mean ± SD	Median (min. - max.)
A (Sham laparotomy, negative control)	8	0.56 ± 0.04	0.32 ± 0.02	0 (0 - 0)
B (Burned, untreated, positive control)	8	1.06 ± 0.34 *	0.45 ± 0.10 *	3 (1 - 4) *
C (Burned, received a single dose of infliximab, an hour after recovery)	7	0.71 ± 0.09 *	0.51 ± 0.16	2 (1 - 2) *
D (Burned, received two doses of infliximab, an hour after recovery and on the 14th day)	8	0.95 ± 0.15 *	0.43 ± 0.09	2 (1 - 4) *

There was no significant difference between Group B and infliximab received Groups C and D in means of hydroxyproline level, stenosis index, and histopathological damage score ($P > 0.05$). SD : Standard Deviation.
* : $P < 0.05$; significant when compared with Group A.

3.1. *Quantity of hydroxyproline*

The quantity of hydroxyproline in the sham laparotomy group was significantly lower compared with the other groups ($P < 0.05$). There was no significant difference between the untreated control Group B and treatment Groups C and D in the quantity of hydroxyproline content ($P > 0.05$ and $P > 0.05$, respectively).

3.2. *Stenosis Index*

The histological slides and the parameters used for the calculation of the stenosis index are shown in Figure 1. The stenosis index in the sham laparotomy group was significantly lower than in the untreated group B ($P < 0.05$). There was no significant difference between the untreated control Group B and treatment Groups C and D in the stenosis index ($P > 0.05$ and $P > 0.05$, respectively).

3.3. *Histopathological damage*

Histopathological damage score in Group B was worse than Group C and Group D (median scores : 3, 2, and 2, respectively). But the difference was statistically not significant between the untreated control Group B and treatment Groups C and D ($P > 0.05$ and $P > 0.05$, respectively). Histopathological slides are shown in Figure 1A and 1B.

4. Discussion

TNF-α is one of the critical factors that mediate inflammatory and immunological response to stress, infection and injury. Its wide range of activities includes the growth-promoting activity on fibroblasts, which leads to increased local collagen deposition (20). TNF-α was reported to be responsible for regulating other mediators that induce inflammation and fibrosis in CCl_4-induced hepatotoxicity (21). Besides, antibodies to TNF-α were reported to prevent the development of concanavalin A induced hepatic fibrosis in a rodent model (22). The importance of TNF-α in sustaining clinical signs of mucosal inflammation is suggested from studies that have demonstrated the efficacy of TNF-α neutralizing therapies both in clinical trials and in animal models (23-25). In irradiation induced oesophageal strictures TNF-α has been demonstrated to increase (26). We therefore hypothesized that inhibition of TNF-α would reduce the development of fibrosis in the healing period of the alkaline oesophageal burns.

Anti-TNF treatments have complex immunomodulatory effects, and may be useful in several inflammatory conditions (27). However this is not true in every aspect since etenarcept, another TNF antagonist with ability to block only soluble TNF, was found effective in rheumatoid arthritis, but not in Crohn's disease (28). Actually in a case with Crohn's disease etenarcept treatment induced obstruction due to stricture development. Authors suspected that acutely decreased inflammation by anti-TNF treatment could worsen stricture (29). Infliximab which is different from etanercept (30) was shown to be effective in inflammatory stenosis of Crohn's disease (31) tough all patients with strictures were excluded from that study. In a recent study reported by Di Sabatino *et al.* the action of infliximab was investigated in colonic myofibroblasts isolated from patients with Crohn's disease (32). In this study infliximab was shown to decrease Crohn's disease myofibroblast collagen production, to enhance tissue inhibitor of metalloproteinases-1 production, and to enhance myofibroblast migration which may facilitate the wound healing (32). The results of our study showed that inhibition of TNF-α using infliximab did not ameliorate the development of fibrosis in alkaline burns of the oesophagus in rats.

The complexity of the cytokine cascade can make precise determination of efficacy of TNF antagonism difficult in experimental models. One of the reasons for this challenge lies in the timing and duration of cytokine antagonism in experimental models. The marked activity of TNF-α was reported to start after 60-90 min of in acute inflammation (33). In this study the first infliximab dosage was given 60 min after the rats completely recovered from anesthesia to resemble the situation on

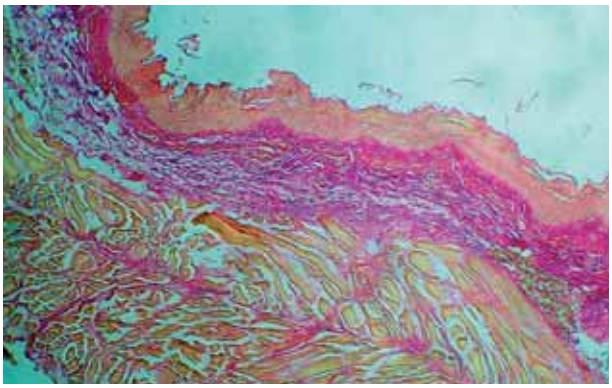

Fig. 1. — (A) Histopathological slides of the oesophagus of an untreated positive control rat (group B).

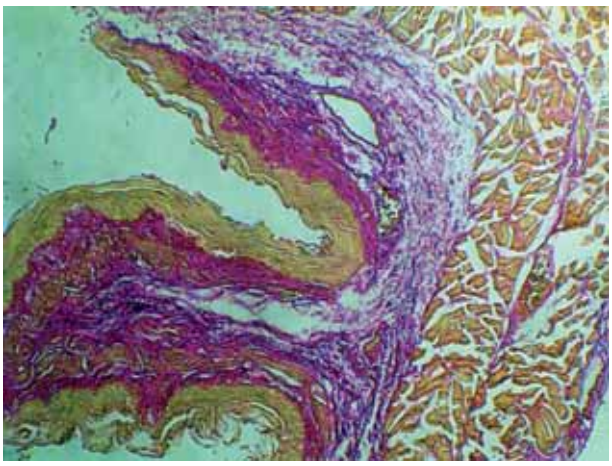

Fig. 1. — (B) Histopathological slides of the oesophagus of a rat given repeated doses of infliximab (group D). Note the thickened submucosa and increase in collagen fibers both in figure A, and B (Van Giesson stain; original magnification ×200).

the field. The clinical benefits of infliximab may be maintained for longer period in repeat doses (34). Therefore one of the study groups (Group C) received infliximab in repeated doses to extend TNF-α blockage. However neither the single nor the repeated doses of infliximab were effective in alkaline burns of oesophagus.

The knowledge about the factors leading to the activation of fibroblasts and myofibroblasts and in turn stricture development in the oesophagus is limited (20). Although TNF-α is generally accepted as a profibrinogenic cytokine, in some reports it was reported to inhibit collagen synthesis (35,36). Transient exposure of fibroblasts to TNF-α inhibits collagen accumulation and functions as a wound healing deactivation signal (37,38). So in case TNF-α was inhibited, it would be expected to lead enhanced collagen production by fibroblasts, but we did not see this relation either. On the other hand, effect of TNF-α on fibroblasts in cultures in vitro not necessarily reflects its effects in vivo. Even within the same tissue the effect of TNF-α on development of fibrosis might be different dependent on the insult. As observed by Bahcecioglu et al who investigated TNF-α and leptin levels in two different liver fibrosis models, and found that TNF-alpha levels and degree of tissue fibrosis were higher in animals with liver fibrosis induced by CCl_4 ; than in animals with liver fibrosis induced by common bile duct ligation (39). So it is difficult to predict the real role of TNF-α in alkaline oesophageal injury, though TNF-α inhibition was not useful. Nevertheless TNF-α may not be the only mediator of inflammation and fibrosis in oesophagus. In chronic oesophagitis IL-1β, TNF-α, MCP-1, MIP-1 α, MIP-2, CINC-2α, and ICAM-1 mRNA expression was significantly increased in oesophageal lesions compared with normal oesophagus (40). Somuncu et al investigated the effects of trapidil, an inhibitor for phosphodiesterase and platelet derived growth factor, in acute phase of corrosive oesophageal injury in a rat model (41). Trapidil, which inhibits platelet aggregation via blockage of thromboxane A2, reduces lipid peroksidation, interleukin 6 and 12. It was shown to decrease neutrophil infiltration in acute phase of corrosive injury and may ameliorate corrosive oesophageal injury and reduce the stricture formation (41). Inflammatory cytokines such as TGF-β1, PDGF, EGF, FGF, IL-1 have been demonstrated to increase the migration of fibroblasts to the site of injury and subsequent proliferation resulting in increased synthesis of collagen and fibronectin, in irradiation induced oesophageal strictures (26). Some of these cytokines have also been demonstrated to stimulate synthesis of extracellular matrix as well as prevent its degradation, which altogether may produce oesophageal stricture after irradiation. In case of alkaline burns of oesophagus inhibition of TNF-α alone might not be effective due to ongoing activities of other cytokines. Targeting and inhibiting those cytokines might be investigated in alkali burns of the oesophagus in further studies.

According to our knowledge, this study is the first in the literature that is searching the effects of inflammatory cytokines in the management of caustic injuries of the oesophagus. In conclusion, although TNF-α is reported to inhibit collagen gene transcription and collagen synthesis in Crohn's disease myofibroblasts, in cultured human fibroblasts and in rat granulation tissue (32,35, 36,42), our results show that anti-TNF-α treatment with infliximab do not demonstrate any beneficial effects in an experimental model of alkaline oesophageal burn in rats. Further evaluation of inflammatory and immunological events leading to stricture in alkaline oesophageal burns, and further studies aiming blockade of other inflammatory cytokines such as TGF-β1, PDGF, EGF, FGF, IL-1 may provide new perspectives for the treatment of alkaline oesophageal burns.

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