The effects of granulocyte colony-stimulating factor on the healing of tracheal anastomosis following radiation therapy in rats

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Abstract

Objective: This study evaluates the effects of granulocyte colony-stimulating factor on the healing of tracheal anastomosis following radiation therapy in rats. Methods: Fifty-six male Wistar rats were divided into four groups. Group 1 underwent tracheal anastomosis. Group 2 underwent radiation therapy followed by tracheal anastomosis. Group 3 underwent radiation therapy followed by tracheal anastomosis and received granulocyte colony-stimulating factor. Group 4 underwent sham radiation therapy followed by sham tracheal anastomosis. At 10 days following radiation therapy, the trachea was dissected for histopathological, mechanical and biochemical evaluation. Results: Median scores for inflammation were three points for Group 1, two points for Group 2, two points for Group 3 and one point for Group 4. Median scores for angiogenesis were four points for Group 1, two points for Group 2, three points for Group 3 and one point for Group 4. Median scores for connective tissue regeneration were four points for Group 1, two points for Group 2, three points for Group 3 and one point for Group 4. Median scores for epithelial regeneration were two points for Group 1, one point for Group 2, one point for Group 3 and one point for Group 4. Mean anastomotic bursting pressures were 853 mmHg for Group 1, 293 mmHg for Group 2, 417 mmHg for Group 3 and 966 mmHg for Group 4. Mean hydroxyproline concentrations were 159 μg/mg for Group 1, 177 μg/mg for Group 2, 120 μg/mg for Group 3 and 117 μg/mg for Group 4. Conclusions: This study suggests that granulocyte colony-stimulating factor contributes to the healing of tracheal anastomosis following radiation therapy through improved connective tissue regeneration.

Keywords: Trachea; Anastomosis; Radiation therapy; Granulocyte colony-stimulating factor; Rats

1. Introduction

For patients with locally advanced non-small-cell lung carcinoma, improved survival relies on neoadjuvant treatment strategies that precede major pulmonary resections. Major pulmonary resections involving the circumferential transection of the trachea require bronchoplastic procedures with end-to-end anastomosis, albeit at the expense of complications regarding connective tissue regeneration in the area of the anastomosis [1]. Connective tissue regeneration is mainly characterized by the stimulation of the fibroblasts towards the accelerated production and release of the immature collagen fibrils with limited crosslinks, followed by the formation of the mature collagen fibrils with abundant crosslinks in the area of the anastomosis [2]. Radiation therapy significantly impedes the fibroblasts in their attempt to maintain the increased demand at mature collagen fibers through decelerating the production as well as the maturation of the collagen fibers through degenerative effects including depleted cellular elements, compromised blood flow and structurally and functionally altered extracellular matrix interactions [3]. Therefore, radiation therapy might impair the tracheal anastomosis with a resultant increase in complications [4]. As a consequence, attempts at improved healing and reduced complications following tracheal anastomosis should intervene with one or more of the interdependent measures of inflammation, angiogenesis, connective tissue regeneration and epithelial regeneration through modulatory effects on the endogenous mediators involved in the healing process such as cytokines and growth factors. Granulocyte colony-stimulating factor is a cytokine produced by the monocytes, the fibroblasts and the endothelial cells with favorable modulatory effects on the endogenous mediators of connective tissue regeneration.
2. Methods and materials

The study was undertaken at the 'Laboratory for Experimental Studies of İnönü University' in compliance with the 'European Convention on Animal Care' following the approval of the design by the 'Animal Ethics Committee of İnönü University'. Fifty-six male Wistar rats (ages between 10 and 12 weeks and weights between 200 and 250 g) were divided into four groups, each consisting of 14 rats. The rats in each group were kept in separate cages in rooms with controlled light and temperature and were fed with standard chow and water ad libitum.

2.1. Study design

The rats in Group 1 underwent tracheal transection and anastomosis. The rats in Group 2 underwent radiation therapy followed by tracheal transection and anastomosis. The rats in Group 3 underwent radiation therapy followed by tracheal transection and anastomosis and received granulocyte colony-stimulating factor. The rats in Group 4 underwent sham radiation therapy followed by sham operation (with no tracheal transection and no anastomosis).

2.2. Radiation therapy

Prior to radiation therapy, the rats received anesthesia using ketamine (Ketalar, Pfizer İlaçları Limited Şirketi, İstanbul, Turkey) at a dose of 80 mg/kg and xylazine (Rompun, Bayer Türk Kimya Sanayi Limited Şirketi, İstanbul, Turkey) at a dose of 5 mg/kg administered using an intraperitoneal injection. The rats were immobilized in the supine position on a rough surface by way of taping the extremities. The operations were performed by the same surgeon who was blinded to the groups. For the rats in Group 1, Group 2 and Group 3, the trachea was exposed through a horizontal skin incision caudal to the level of the larynx after dissection and retraction of the strap muscles and was dissected free from the surrounding tissues in its entirety. Tracheal transection was undertaken between the third and the fourth rings over the majority of the circumference in attempt to facilitate re-approximation. The tracheal continuity was restored by an end-to-end anastomosis using four interrupted sutures of 6-0 polypropylene (Medilen, Medeks Anonim Şirketi, İstanbul, Turkey). Following re-approximation of the strap muscles, the skin incision was closed using interrupted sutures of 3-0 polypropylene (Medilen, Medeks Anonim Şirketi, İstanbul, Turkey). For the rats in Group 4, the trachea was exposed through a horizontal skin incision caudal to the level of the larynx and the skin incision was closed using interrupted sutures (with no tracheal transection and no anastomosis). Following the operations, the animals were closely observed until recovery from anesthesia.

2.3. Tracheal anastomosis

Prior to tracheal transection and anastomosis, the rats received anesthesia using ketamine at a dose of 80 mg/kg and xylazine at a dose of 5 mg/kg administered using an intraperitoneal injection. The rats were immobilized in the supine position on a rough surface by way of taping the extremities. The operations were performed by the same surgeon who was blinded to the groups. For the rats in Group 1, Group 2 and Group 3, the trachea was exposed through a horizontal skin incision caudal to the level of the larynx after dissection and retraction of the strap muscles and was dissected free from the surrounding tissues in its entirety. Tracheal transection was undertaken between the third and the fourth rings over the majority of the circumference in attempt to facilitate re-approximation. The tracheal continuity was restored by an end-to-end anastomosis using four interrupted sutures of 6-0 polypropylene (Medilen, Medeks Anonim Şirketi, İstanbul, Turkey). Following re-approximation of the strap muscles, the skin incision was closed using interrupted sutures of 3-0 polypropylene (Medilen, Medeks Anonim Şirketi, İstanbul, Turkey). For the rats in Group 4, the trachea was exposed through a horizontal skin incision caudal to the level of the larynx and the skin incision was closed using interrupted sutures (with no tracheal transection and no anastomosis). Following the operations, the animals were closely observed until recovery from anesthesia.

2.4. Granulocyte colony-stimulating factor

For the rats in Group 3, granulocyte colony-stimulating factor (Neupogen, Roche Müşahararlari Sanayi Anonim Şirketi, İstanbul, Turkey) was administered using a subcutaneous injection at a dose of 100 μg/kg per day for five consecutive days starting on the day of the operation. For the rats in Group 4, 0.9% sodium chloride at the same volume with granulocyte colony-stimulating factor was administered using a subcutaneous injection for five consecutive days starting on the day of the sham operation.

2.5. Euthanasia

The rats underwent euthanasia at 10 days following radiation therapy. Prior to euthanasia, the rats received anesthesia using propofol (Propofol, Abbott Laboratuvarı Anonim Şirketi, İstanbul, Turkey) at a dose of 50 mg/kg administered using an intraperitoneal injection. Euthanasia was performed by way of transcardiac perfusion using 0.9% sodium chloride.

2.6. Histopathological evaluation

For seven rats in each group, the trachea was dissected starting caudally, trimmed to leave a section containing the area of transection and anastomosis with one additional ring cranially and caudally, placed in formaldehyde, embedded in paraffin, sliced into 5 μm thick sections and stained using haematoxylin and eosin and Masson’s trichrome. Histopathological evaluation was performed by the same pathologist, who was blinded to the groups, under the light microscope (BX50, Olympus Corporation, Tokyo, Japan) using a grid system. Among items descriptive for anastomotic healing, inflammation (defined as the presence of vascular dilatation with interstitial fluid and leukocyte accumulation), angiogenesis (defined as the presence of immature vasculature outgrowing from the mature vasculature) and connective tissue regeneration (defined as the presence of fibroblast and
collagen accumulation) were semi-quantitatively scored as not increased (0 point), mildly increased (1 point), moderately increased (2 points) or significantly increased (3 points) and epithelial regeneration (defined as the extent of epithelial closure) was semi-quantitatively scored as complete disruption (0 point), partial closure (1 point) or complete closure (2 points) [7].

2.7. Mechanical and biochemical evaluation

For seven rats in each group, the trachea was dissected starting caudally, trimmed to leave a section containing the area of transection and anastomosis with two additional rings cranially and caudally and placed in 0.9% sodium chloride. A catheter was inserted cranially and a manual pressure transducer (Model 50-8952, Harvard Apparatus Incorporation, South Natick, United States of America) was inserted caudally into the trachea. The transducer was tied in position using sutures of 2-0 silk (Sterisilk, Steril Sağlık Malzemeleri Sanayi ve Ticaret Anonim Şirketi, İstanbul, Turkey) and 0.9% sodium chloride was infused into the catheter. The anastomotic bursting pressure was determined by the same researcher, who was blinded to the groups, using an oscillograph (Model 60-9315, Harvard Apparatus Incorporation, South Natick, United States of America) and was expressed in mmHg.

Subsequently, the trachea and the standard hydroxyproline (cis-4-hydroxy-L-proline, Sigma–Aldrich Corporation, St. Louis, United States of America) were placed in separate hydrolysis tubes, hydrolyzed at 110 °C with diluted sodium hydroxide and oxidized at 22 °C with chloramine-T reagent. The Ehrlich’s reagent was added to the tubes and the color was allowed to develop at 60 °C. The hydroxyproline concentration of the trachea was determined by the same researcher, who was blinded to the groups, using the absorbance value at 560 nm on a spectrophotometer (Model UV-1240, Shimadzu Corporation, Kyoto, Japan), plotted against the standard hydroxyproline concentration and expressed in μg/mg (micrograms per dry weight of the trachea in milligrams) [8].

2.8. Statistical analysis

Median item scores of histopathological evaluation were compared using the chi-square test. Mean anastomotic bursting pressures and mean hydroxyproline concentrations were compared using the one-way analysis of variance (ANOVA). Statistical analysis was performed using the ‘10.0’ version of the ‘SPSS for Windows’ software package. Statistical significance was defined as the P-value being less than or equal to 0.05.

3. Results

At the time of tracheal transection and anastomosis, failure to restore the tracheal continuity resulted in severe respiratory distress and death in two of the rats in Group 1 and one of the rats in Group 2. The tracheas of these rats were not available for evaluation. All of the remaining rats survived until the time of euthanasia. At the time of euthanasia, an abscess formation was observed in one of the rats in Group 1 and a fistula formation was observed in two of the rats in Group 2. The tracheas of these rats were not available for evaluation.

3.1. Histopathological evaluation

Median scores for inflammation were three points for Group 1, two points for Group 2, two points for Group 3 and one point for Group 4. Regarding inflammation, the difference between the groups was statistically significant (P = 0.001, χ² = 22.47) (Fig. 1). Median scores for angiogenesis were four points for Group 1, two points for Group 2, three points for Group 3 and one point for Group 4. For angiogenesis, the difference between the groups was statistically significant (P < 0.001, χ² = 39.84) (Fig. 2). Median scores for connective tissue regeneration were four points for Group 1, two points for Group 2, three points for Group 3 and one point for Group 4. Regarding connective tissue regeneration, the difference between the groups was statistically significant (P = 0.007, χ² = 17.78) (Fig. 4).

3.2. Mechanical and biochemical evaluation

Mean anastomotic bursting pressures were 853 mmHg (ranging from 760 to 920 mmHg) for Group 1, 293 mmHg

![Fig. 1. In Group 1, inflammation was pronounced following the tracheal anastomosis (a). When radiation therapy preceded the tracheal anastomosis in Group 2, inflammation was partially depressed (b). The administration of granulocyte colony-stimulating factor in Group 3 did not contribute to the restraint of inflammation (c) (Masson’s trichrome, 200x).](https://academic.oup.com/ejcts/article-abstract/30/6/840/366148)
Fig. 2. The tracheal anastomosis was associated with the enhanced angiogenic response in Group 1 (a). The angiogenic response was markedly diminished when radiation therapy preceded the tracheal anastomosis in Group 2 (b). Through the administration of granulocyte colony-stimulating factor, the enhanced angiogenic response was partially restored in Group 3 (c) (Masson’s trichrome, 40×).

Fig. 3. In Group 1, connective tissue regeneration was accelerated following the tracheal anastomosis (a). When radiation therapy preceded the tracheal anastomosis in Group 2, connective tissue regeneration was markedly impeded (b). Through the administration of granulocyte colony-stimulating factor, connective tissue regeneration was partially improved in Group 3 (c) (Masson’s trichrome, 100×).

Fig. 4. Epithelial regeneration was promoted following the tracheal anastomosis in Group 1 (a). Epithelial regeneration was partially impeded when radiation therapy preceded the tracheal anastomosis in Group 2 (b). The administration of granulocyte colony-stimulating factor in Group 3 did not contribute to the improvement of epithelial regeneration (c) (hematoxylin and eosin, 400×).

Fig. 5. Anastomotic bursting pressures by groups are expressed as range and mean (Group 1: tracheal anastomosis, Group 2: radiation therapy followed by tracheal anastomosis, Group 3: radiation therapy followed by tracheal anastomosis and granulocyte colony-stimulating factor, Group 4: sham radiation therapy followed by sham tracheal anastomosis).

Fig. 6. Hydroxyproline concentrations by groups are expressed as range and mean (Group 1: tracheal anastomosis, Group 2: radiation therapy followed by tracheal anastomosis, Group 3: radiation therapy followed by tracheal anastomosis and granulocyte colony-stimulating factor, Group 4: sham radiation therapy followed by sham tracheal anastomosis).
Granulocyte colony-stimulating factor is produced by the cells involved in the healing process, namely the monocytes, the fibroblasts and the endothelial cells, and acts on the granulocyte restricted progenitor cells in the bone marrow, promoting the proliferation and release of the granulocytes besides enhancing their functional activity regarding phagocytic and bacteriocidal potentials [5]. Moreover, granulocyte colony-stimulating factor augments the mobilization of the endothelial progenitor cells from the bone marrow, thus contributing to the angiogenic response [9]. Therefore, granulocyte colony-stimulating factor accelerates the healing process by restraining inflammation [10], restoring angiogenesis [11] and improving connective tissue regeneration and epithelial regeneration [12].

Regarding the healing process, inflammation corresponds to the accomplishment of homeostasis and the formation of a temporary extracellular matrix to enable the active migration of the monocytes and the fibroblasts. Transforming growth factor beta, a cytokine produced by the cells involved in the healing process, is critically required in the transition from inflammation towards connective tissue regeneration through the demotion of the active migration of the monocytes and the promotion of the proliferation and differentiation of the fibroblasts [13]. Starting immediately following the insult and resolving within 3—4 days, inflammation seems to be the most vulnerable event as far as the deleterious effects of radiation therapy on the healing process are concerned. Radiation therapy significantly depresses the early changes involved in inflammation during the healing process, thus delaying the transition towards connective tissue regeneration [3]. Transforming growth factor beta, on the other hand, promotes the recruitment, proliferation and differentiation of the fibroblasts despite the absence of the monocytes in the area of healing, hence augmenting the healing impaired by radiation therapy [14]. Granulocyte colony-stimulating factor triggers an imbalance in the production of the endogenous mediators involved in the healing cascade in attempt to counterbalance the impairing effects of radiation therapy on the healing process, presumably leading to the increased production of transforming growth factor beta to generate a state of depressed inflammation [15].

The healing of the tracheal anastomosis is closely related to the regional mucosal blood flow. Among neoadjuvant treatment strategies, radiation therapy significantly reduces the regional mucosal blood flow, leading to early changes adversely affecting the anastomotic healing including reactive congestion, edema and inhibition of capillary proliferation [16]. Therefore, attempts at improved healing have mainly focused on the reinforcement of the irradiated anastomotic segment with pedicled viable tissue to promote the regional mucosal blood flow [17]. Newly formed microvessels improve the microperfusion around the irradiated anastomotic segment, hence the enhancement of the angiogenic response is a valuable management option regarding the impaired anastomotic healing following radiation therapy [18]. The potential for granulocyte colony-stimulating factor to participate in the angiogenic response that appears to be mediated by the release of angiogenic growth factors such as vascular endothelial growth factor and fibroblast growth factor has recently been demonstrated in the ischemic cerebral tissue [11] through the mobilization of the endothelial progenitor cells from the bone marrow, and presumably through the activation of the endothelial cells in the ischemic tissues as well.

Throughout the healing process, connective tissue regeneration commences even prior to the completion of inflammation. Connective tissue regeneration is mainly characterized by the stimulation of the fibroblasts towards the accelerated production and release of the immature collagen fibrils with limited crosslinks, followed by the formation of the mature collagen fibers with abundant crosslinks and the increased deposition of the mature collagen fibers in the anastomotic segment enhances the mechanical strength of the anastomosis [2]. Radiation therapy significantly impedes the fibroblasts in their attempt to maintain the increased demand at mature collagen fibers involving the healing process through decelerating the production as well as the maturation of the collagen fibers [3]. Granulocyte colony-stimulating factor augments the release of transforming growth factor beta and platelet-derived growth factor that, in turn, act on the fibroblasts even in the absence of the monocytes to improve connective tissue regeneration through the acceleration of the formation of the mature collagen fibers [6].

Following connective tissue regeneration, the healing process is wrapped up with epithelial regeneration that is accomplished through the migration of the keratinocytes from the edges of the area of the insult towards the temporary extracellular matrix over the basement membrane and their proliferation thereafter [19]. Radiation therapy ruins epithelial regeneration through the inhibition of the migrative and proliferative capabilities of the keratinocytes [3]. Granulocyte colony-stimulating factor is involved in the institution of epithelial regeneration through the promotion of the proliferation of the epithelial stem cells as well as the keratinocytes, presumably mediated by the release of keratinocyte growth factor [20].

Regarding the hyaline cartilage framework of the trachea, the mature collagen fibers of the extracellular matrix are responsible for the mechanical strength. Following anastomotic procedures, the mechanical strength might be evaluated using either the anastomotic bursting pressure that reflects the resistance of the area of the anastomosis or the hydroxyproline concentration that reflects the integrity
of the area of the anastomosis [21]. The anastomotic bursting pressure is associated with the quality of the mature collagen fibers with abundant crosslinks rather than the quantity of the immature collagen fibrils with limited crosslinks in the area of the anastomosis [22]. Therefore, arguing against the use of the hydroxyproline concentration as an exclusive measure of the mechanical strength, the anastomotic bursting pressures and the hydroxyproline concentrations are not undoubtedly correlated [23]. Following anastomotic procedures, the mechanical strength allegedly parallels the formation of the mature collagen fibers, gradually increasing over 7 days [24]. Besides the mature collagen fibers, the mechanical strength might be influenced by the surgeon performing the tracheal anastomosis as well as the sutures used to perform the tracheal anastomosis [25].

In this study, the tracheal anastomosis was performed using identical sutures for all of the rats by the same surgeon, enabling the unprejudiced evaluation of the individual effects of radiation therapy and granulocyte colony-stimulating factor on the healing of tracheal anastomosis. Granulocyte colony-stimulating factor was administered for five consecutive days starting on the day of the tracheal anastomosis in attempt to stimulate the fibroblasts towards the accelerated formation of the mature collagen fibers. The enhanced angiogenic response associated with the tracheal anastomosis was markedly diminished when radiation therapy preceded the tracheal anastomosis, whereas the angiogenic response was partially restored through the administration of granulocyte colony-stimulating factor. The accelerated connective tissue regeneration following the tracheal anastomosis was markedly impeded when radiation therapy preceded the tracheal anastomosis, whereas through the administration of granulocyte colony-stimulating factor connective tissue regeneration was partially improved. The anastomotic bursting pressures established following tracheal anastomosis were markedly lower when radiation therapy preceded the tracheal anastomosis, whereas through the administration of granulocyte colony-stimulating factor connective tissue regeneration was partially improved. The anastomotic bursting pressures were slightly higher through the administration of granulocyte colony-stimulating factor. The hydroxyproline concentrations established following tracheal anastomosis were slightly higher when radiation therapy preceded the tracheal anastomosis, whereas the hydroxyproline concentrations were markedly lower through the administration of granulocyte colony-stimulating factor.

In conclusion, this study suggests that granulocyte colony-stimulating factor contributes to the healing of tracheal anastomosis following radiation therapy through improved connective tissue regeneration, acting on the fibroblasts to accelerate the formation of the mature collagen fibers. The representation of the formation of the mature collagen fibers in the anastomotic bursting pressures in contrast to the representation of the degradation of the mature collagen fibers in the hydroxyproline concentrations clarifies the discordance between the anastomotic bursting pressures and the hydroxyproline concentrations. In this respect, the mature collagen fibers in the area of the anastomosis appear to make the essential contribution towards the enhanced mechanical strength of the hyaline cartilage framework of the trachea.

References