Local Angioneogenetic Effect of Intramuscular Interleukin-8 Injection

İntramusküler İnterlökin-8 Enjeksiyonunun Lokal Anjiyoneogenetik Etkisi

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ABSTRACT Objective: Angioneogenesis, which plays significant roles in a variety of physiological processes such as embryonic growth and wound healing, is strictly delimited and is finely tuned by a balance of proangiogenic and antiangiogenic factors. This study was conducted to investigate the angioneogenic effect of interleukin-8 (IL-8) administered intramuscularly. **Material and Methods:** Twelve New Zealand white rabbits were included in this study. A total daily dose of 4 micrograms (1 mcg/kg) of IL-8 was administered into the left (Group A) and saline solution into the right (Group B) gluteus maximus muscles of 6 rabbits for 6 days. The remaining 6 rabbits constituted the sham group (Group C). Gluteal muscle samples were obtained from injection sites in all groups and were stained with hematoxylin-eosin and immunohistochemically by using streptavidin biotin method with CD31 antibody. Avidin-biotin peroxidase method (LSAB) was used as secondary and binding antibody. Diaminobenzidine tetrahydrochloride (DAB) was used as chromogenic substance. In immunohistochemical staining with CD31, vascular channels covered with brown stained cells or cell clusters were considered and were counted as vascular network. **Results:** Three subjects in Group A and one subject in Group B displayed findings of large muscle necrosis and regeneration. Vascular channel score was significantly higher in Group A (p=0.032) (Group A; median=12.5, min=6, max=16. Group B; median=5, min=4, max=13. Group C; median=4.5, min=4, max=13.) than in the other groups. **Conclusion:** IL-8 chemokine family seems to stimulate angioneogenesis in rabbit skeletal muscles. This result is promising in terms of the possible therapeutic potential of IL-8. Daily administration at a dose of around 1 mcg/kg caused local tissue necrosis, hence use of alternative routes such as intraarterial administration must be investigated to avoid such complications.

Key Words: Angioneogenesis; interleukin-8; skeletal muscle

ÖZET Amaç: Embriyonik büyüme ve yara iyileşmesi gibi bir dizi fizyolojik süreçte önemli rol oynayan anjiyogenez, proanjiyojenik ve antianjiyojenik faktörlerin birbirlerinden kesin çizgiler ile dengelenmesiyle ayrılmış ve hassas şekilde düzenlenmiştir. Bu çalışma, intramusküler olarak uygulanan interlökin-8 (IL-8)'in anjiyoneogenetik etkisini araştırmak için yapılmıştır. **Gereç ve Yöntemler:** Çalışmada on iki adet Yeni Zelanda cinsi beyaz tavşan kullanıldı. Altı gün boyunca altı tavşanın sol gluteusuna 1 mcg/kg'dan toplam 4 mikrogram IL-8 (Grup A), sağ gluteusuna serum fizyolojik (Grup B) enjeksiyonu uygulandı. Diğer altı tavşan kontrol grubu idi (Grup C). Enjeksiyon uygulanan bölgelerden alınan gluteal kas örnekleri, Hemotoksilen-Eozin ve Streptavidin-biotin yöntemi kullanılarak CD31 antikoru ile immünohistokimyasal olarak boyandı. Avidin-biotin peroksidaz metodu (LSAB) ikincil ve bağlayıcı antikor olarak kullanıldı. Kromojen madde olarak ise diaminobenzidin tetrahidroklorit (DAB) kullanıldı. CD31 ile yapılan immünohistokimyasal boyamada kahve renkli olarak boyanmış hücreler veya hücre grupları, endotel ile döşeli yeni vasküler kanallar, ağ (vascular network) olarak kabul edilerek sayıldı. **Bulgular:** Diğer gruplarla karşılaştırıldığında, A Grubu'nda vasküler kanal skoru anlamlı düzeyde daha fazla saptandı (p=0,032). (Grup A; ortanca=12,5, min=6, maks=16. Grup B; ortanca=5, min=4, maks=13. Grup C; ortanca=4,5, min=4, maks=13.). **Sonuç:** Bu çalışma, iskelet kasında IL-8 ile anjiyoneogenezin stimüle edilebileceğini göstermiştir. IL-8'in 1 mcg/kg dozunda lokal olarak uygulanması halinde kas nekrozu gelişme riski bulunması nedeniyle, IL-8'in arter içine uygulanması gibi alternatif yöntemlere yönelik ileri araştırma yapılması yararlı olabilir.

Anahtar Kelimeler: Anjiyoneogenez; interlökin-8; iskelet kası

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Interleukin-8 (IL-8) is a member of the chemokine family that plays a major role in the pathogenesis of inflammation, infection, tissue damage, allergy, cardiovascular diseases and tumor growth. IL-8 affects all blood cells and endothelium in terms of chemotaxis, adhesion, excretion of granular contents like superoxide and histamine, mitogenesis and angiogenesis.[1-6]

IL-8 stimulates organization of chronic inflammation and angiogenesis, which describes penetration of new blood vessels into the area of inflammation and their development following tissue healing.[1-6]

IL-8 was reported to act on angiogenesis in the pathogenesis of tumors like malignant melanoma, non-small cell lung carcinoma and ovarian cancer. There are ongoing studies to develop therapeutic strategies for this condition.[7-9]

The hypothesis of our study is that therapeutic doses of IL-8 stimulate angioneogenesis in the skeletal muscle tissues of rabbits.

MATERIAL AND METHODS

The experimental procedure was in accordance with the "Position of the American Heart Association on Research Animal Use". Animal care complied with the 'Principles of Laboratory Animal Care' as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 5377-3, 1996). The experimental study was approved by the Animal Research Ethics Committee of Rize University Medical Faculty (Reference Number: 2011/16). All animals were given 5 days of adaptation to their environment prior to experiments. The room temperature was kept between 28-30°C.

EXPERIMENTAL PROTOCOL

Twelve New Zealand white rabbits were included in this study. Gluteal regions of the rabbits were shaved. For 6 days, one gluteus maximus muscle was injected daily with 1 mcg/kg of IL-8 (Biovision recombinant human endotelial IL-8, 4149-25 cat number) (Group A) and the contralateral gluteus maximus muscle was injected with saline solution (Group B) at the same depth. The remaining 6 rabbits constituted the sham group (Group C). At the end of day 7, these rabbits were sacrificed and specimens of 1.5x1.5x0.5 cm were obtained from both gluteal muscle regions.

HISTOPATHOLOGIC ASSAY

These specimens were fixed within 10% formaldehyde solution for 24 hours. Fixed tissues underwent routine tissue processing. Slices of 5-μm thickness were obtained from paraffin blocks and were placed on polylysine-coated slides. One of the slices was stained with hematoxylin-eosin and the other immunohistochemically with CD31 antibody using the streptavidin biotin method (Lab Vision, PECAM-1, clone 1A10). Avidin-biotin peroxidase (LSAB, Dako, Denmark) was used as secondary and binding antibody. Diaminobenzidine tetrahydrochloride (DAB) was used as chromogenic substance. In immunohistochemical staining with CD31, vascular channels covered with brown stained cells or cell clusters were considered and counted as vascular network (Figures 1a, b). Counting procedure was conducted by two blinded pathologists at different times. The pathologists counted 3 large magnification zones (x400; Nikon E400) with the most intense positivity and calculated their average score. For every single experimental animal, the mean value of the results of two pathologists were considered (decimal numbers were rounded up).

STATISTICAL ANALYSES

Nonparametric data were expressed as median (min-max). SPSS 16.0 (SPSS, Inc., Chicago, Illinois) was used to perform statistical analyses. The three groups were compared with the Kruskal Wallis tests. Mann-Whitney U test was used to compare the two independent groups of sampled data. A p value of 0.05 was considered statistically significant.

RESULTS

Three subjects in Group A and one subject in Group B displayed findings of muscle necrosis and regeneration. Median vascular network scores ob-

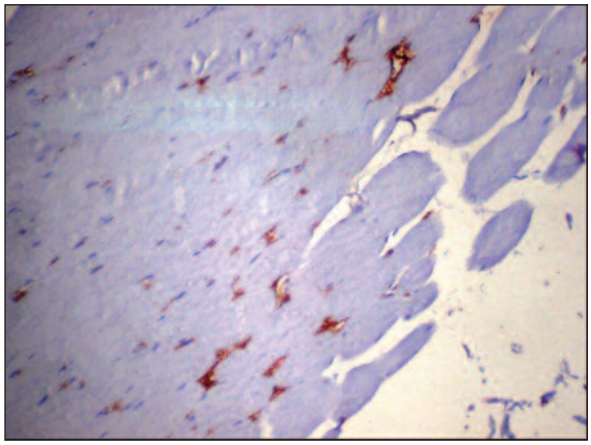

FIGURE1A: Vascular network stained with CD31 in skeletal muscle of a subject from the control group (x100, DAB).
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

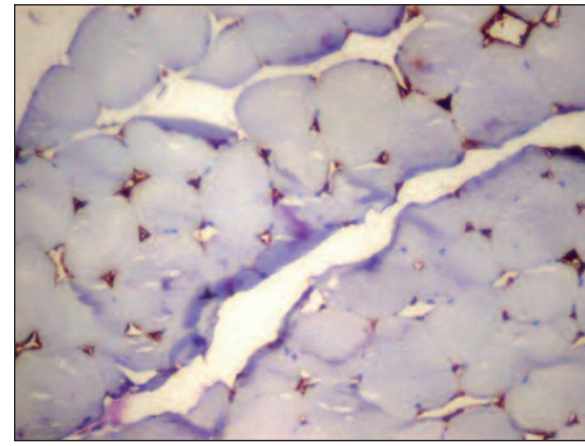

FIGURE 1B: Vessels covered with CD31-positive endothelium in the skeletal muscle of a subject from the IL-8 group (x100, DAB).
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TABLE 1: Vascular network scores of the groups.

IL-8 Group (n:6)	Saline Group (n:6)	SHAM Group (n:6)	p *
Vascular network scores	12.5 (6-16)	5 (4-13)	4.5 (4-13)
Median (min-max)			

* Kruskal Wallis test.

tained by CD31 positivity were 12.5 (6-16); 5 (4-13) and 4.5 (4-13) in Groups A, B and C, respectively (Table 1) (Figure 2). Statistical analysis with the Kruskal-Wallis test revealed that group A had significantly higher scores for vascular network than the other two groups (p=0.032).

DISCUSSION

All the scientists that are interested in vascular issues aim to either completely solve or at least control the steps in angioneogenesis. It is obvious that if any agent controlling angioneogenesis could have been discovered, this would open a new era in the treatment of ischemic vascular diseases. We aimed to investigate the angioneogenetic effect of IL-8 on rabbit skeletal muscles when administered at therapeutic doses.

Our findings confirmed our initial hypothesis stating that IL-8 would stimulate angioneogenesis in rabbit skeletal muscle tissue. On histopathological examination, regenerated structures and endothelium covered vascular connections were

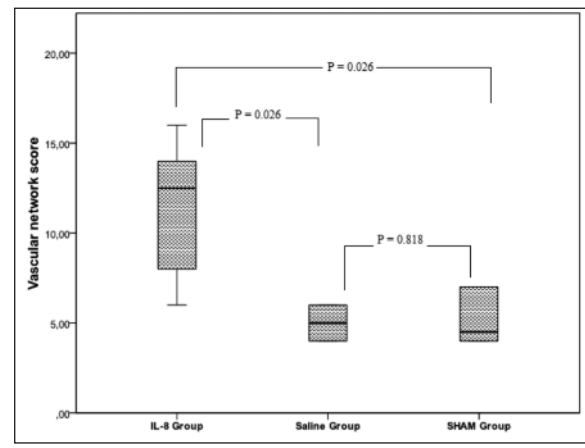

FIGURE 2: Comparison of vascular network score between the IL-8, Saline and Sham groups.

demonstrated and the number of new vascular structures was significantly higher in the study group compated to the other groups (p=0.032). Since the contribution of necrosis on the occurrence of angioneogenesis is difficult to elucidate, the role of IL-8 on angioneogenesis cannot be clarified.

Angioneogenesis is induced with hypoxia in adulthood. In hypoxic conditions, vascular endothelial growth factor (VEGF) is released from the endothelial cells. VEGF increases angioneogenesis by stimulating monocytes in the circulation.[5] Besides, IL-8 originates from monocytes. It is a member of the chemokine family, which provides chemotaxis, adhesion, excretion of granular contents like superoxide and histamine, mitogenesis and angiogenesis by affecting neutrophils, basophils and endothelial cells.[1] A number of studies have addressed the effect of IL-8 on cancer pathophysiology. IL-8 was reported to play a role in the angioneonesis of ovarian and non-small cell lung cancers.[7-9] IL-8 was also suggested to be a potent pro-angiogenic factor that stimulates angioneogenesis by inducing capillary tube formation and endothelial cell proliferation.[4-6,10,11] IL-8 is a locally secreted and acting substance. Basal IL-8 production is low in many organs. It appears rapidly when mRNA level exceeds 1% of total cellular RNA level. IL-8 stabilizes mRNA, thus controlling all the stages of transcription and posttranscription that are necessary for gene regulation.[11,12]

IL-8 secretion increases more prominently in ischemic-, toxic- or inflammatory lesions. Tumor necrosis factor-alpha (TNF-α), lipopolysaccharides, some growth factors as platelet derived growth factor (PDGF), viral infectious agents and secretion of bacterial products are among some stimulants.[11,12]

Angioneogenesis is a process where organization of chronic inflammation and ongoing tissue repair take place and new blood vessels migrate into the inflammatory area and grow. This process depends on the type of chemokine that takes part. Immune protein-10 (IP-10) and platelet factor-4 (PF-4) of the non-ELR CTX (glutamic acid-leucine-arginine chemokine) group inhibit angioneogenesis, whereas IL-8 and growth regulated oncogene-alpha (GRO-alpha) of the ELR-chemokines stimulate angioneogenesis.[1] The balance between these chemokines within the inflamed tissue decides for the new vessel formation.[1,11,13]

Engelhardt et al. investigated the mechanism of wound healing in human beings and saw that the levels of chemokines such as IL-8, IP-10 and monocyte chemotactic protein-1 (MCP-1) changed during the wound healing process. On the first day of injury, particularly IL-8 is secreted at maximal levels activating wound healing by enhancing neutrophilic infiltration on surface.[14] Similarly, Devalaraja *et al.* showed delay in every step of the wound healing in CXCR2 gene-deficient mice. They emphasized the importance of chemoattractants such as IL-8 in epithelization.[15]

Regarding the inhibitory effect of some chemokines on hematopoiesis and proliferation of epithelial progenitor cell, the only proven report is on non-small cell lung carcinoma where IL-8 inhibits tumoral growth *in vitro*.[7,8] In addition, IL-8 was shown to play an important role in ovarian cancer angiogenesis.[9] Kunz et al. suggested that IL-8 had a significant organizing role in the growth of malignant melanoma. They identified that IL-8 activated transcription of mRNA and increased the aggressiveness potential of the tumor *in vivo*.[16]

IL-8, which has a powerful chemotactic effect on neutrophils, has increased levels in the vitreous humor in circumstances such as intraocular inflammation and diabetic retinopathy.[17] Human recombinant IL-8 was shown to enhance neovascularization in rabbit cornea.[12,17]

Onaratti et al. showed that perioperative level of serum IL-8 would rise up to 200 pg/mL among patients not receiving corticosteroids.[18] This potential might be useful as a reserve in angiogenesis capacity. Retsky et al. suggested that angiogenesis could be induced after tumoral surgery.[19] This may depend on the tissue response to surgery. The excised tumor mass may also be effective in this situation. However, the inflammatory processes should also be considered. Göksu Erol et al. reported the relationship between mast cells and angiogenesis.[20] In addition, current studies reported that the effects of the inflammatory events were related with angiogenesis.

The main limitation of the study was the lack of literature information about the appropriate IL-8 dose and its administration time. Contribution of necrosis to the angioneogenesis and lack of vascular scores of subjects that had undergone necrosis

are other limitations of the study. Further investigations are needed regarding this issue.

CONCLUSION

In conclusion, IL-8 chemokine family seems to stimulate angioneogenesis in rabbit skeletal muscle tissue. This result is promising in terms of the possible therapeutic potential of IL-8. Daily administration at a dose of around 1 mcg/kg caused local tissue necrosis; thus, use of alternative routes such as intraarterial administration must be investigated to avoid such complications.

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