


## Effects of Angiostatin on *in vitro* Embryonic Rat Development

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### Summary

The progression of angiogenesis is controlled by a delicate balance between the positive and negative regulators. Angiostatin has potent antiangiogenic effect on endothelial cells, influencing their proliferation, differentiation and other functions. In this study, *in vitro* effects of angiostatin on total embryonic growth were investigated in rat embryos. The rat embryos were explanted on day 9.5 and cultured in whole rat serum (WRS) (for control) and adding 0.5, 2.5 and 5 µg/ml angiostatin (for experimentals) in WRS. After 48 hours culture period, the embryos from each group were harvested and analysed morphologically. The results showed that the embryonic growth and development during organogenesis decreased in the presence of angiostatin when compared to embryos grown in WRS. Mean morphological scores for the embryos grown in WRS and in the presence of 0.5, 2.5 and 5 µg/ml angiostatin were 59.8±2.02, 34.8±8.52, 22.4±6.41 and 17.8±6.19, yolk sac diameters were 6.0±0.2, 4.8±0.55, 4.5±0.57 and 4.2±0.39 mm, crown-rump lengths were 5.4±0.23, 4.4±0.42, 4.1±0.87 and 3.7±0.51 mm and mean protein contents of embryos were 140.76±12.21, 112.66±8.67, 89.85±10.89 and 53.77±8.15 µg/ml respectively. Median value of the somite numbers was 24 (24-25) in control group and it was diminished 14 (12-14), 12 (11-17) and 13 (8-17) respectively in experimental groups. As a result the angiostatin could cause developmental retardation of embryo because of its antiangiogenic effect.

**Keywords:** Angiostatin, Culture, Embryo, *in vitro*, Rat

## Anjiostatinin *in vitro* Embriyonik Rat Gelişimi Üzerine Etkileri

### Özet

Anjiogenez süreci anjiogenez inhibitör ve aktivatörleri arasındaki denge ile kontrol edilmektedir. Anjiostatin endotel hücrelerin çoğalmasını, farklılaşmasını ve diğer fonksiyonlarını etkileyerek endotel hücreleri üzerinde antiangiogenik etki gösterir. Bu çalışmada, anjiostatin'in sıçan embriyolarında *in vitro* total embriyonik gelişim süreci üzerine etkileri araştırıldı. 9.5 günlük embriyolar normal sıçan serumunda (kontrol grubu) ve normal sıçan serumuna 0.5, 2.5 ve 5 µg/ml anjiostatin eklenerek (deney grupları) kültüre edildi. 48 saatlik kültür periyodu sonunda tüm gruplardaki embriyolar morfolojik olarak değerlendirildi. Deney grupları kontrol grubu ile karşılaştırıldığında organogenez süresince embriyonik gelişim ve büyümede bir gerilemenin varlığı gözlemlendi. Sıçan serumu ve sıçan serumu+0.5, 2.5 ve 5 µg/ml anjiostatin'de kültür edilen embriyolarda ortalama morfolojik skor sırasıyla 59.8±2.02, 34.8±8.52, 22.4±6.41 ve 17.8±6.19, yolk sac çapı 6.0±0.2, 4.8±0.55, 4.5±0.57 ve 4.2±0.39 mm, baş-kıç uzunluğu 5.4±0.23, 4.4±0.42, 4.1±0.87 ve 3.7±0.51 mm ve ortalama protein içeriği 140.76±12.21, 112.66±8.67, 89.85±10.89 ve 53.77±8.15 µg/ml olarak belirlendi. Ortalama somit sayısı kontrol grubunda 24 (24-25) iken deney gruplarında azalmaktaydı ve sırasıyla 14 (12-14), 12 (11-17) ve 13 (8-17) olarak hesaplandı. Sonuçlar, anjiostatin'in antiangiogenik etkisine bağlı olarak embriyonik gelişimde bir gerilemenin olabileceğini ortaya koymaktadır.

**Anahtar sözcükler:** Anjiostatin, Kültür, Embriyo, *in vitro*, Sıçan

## INTRODUCTION

During embryogenesis, the formation of blood vessels proceeds by both vasculogenesis and angiogenesis. Both

processes appear to be finely regulated. To date, factors and genes involved in the negative regulation of embryonic



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vasculogenesis remain largely unknown<sup>1</sup>. Angiostatin that acts as an inhibitor of angiogenesis was isolated from both serum and urine of mice bearing a transplantable murine Lewis Lung carcinoma (3LL) in syngeneic C57B16/J mice. Proteolytic fragments of human plasminogen comparable to the murine angiostatin, but not intact plasminogen, inhibit the neovascularization and growth of lung metastases in mice. Angiostatin can be generated *in vitro* by limited elastase proteolysis of plasminogen. Studies with protease inhibitors have demonstrated that a serine protease is essential for angiostatin generation. Studies of smaller fragments of human angiostatin on inhibition of endothelial proliferation have demonstrated that functional difference is present among individual kringle structures<sup>2-4</sup>. Angiostatin with one less kringle molecule (kringle 1 to 3) has recently been demonstrated to be an effective angiogenic inhibitor<sup>5</sup>. The *in vitro* activity of angiostatin in endothelial cell proliferation assays resides in kringles 1-3, with kringle 1 being the most potent inhibitor and kringle 4 being relatively ineffective<sup>3,4,6</sup>. However the mechanism underlying how angiostatin and its related kringle fragments specifically inhibit endothelial cell growth is unclear<sup>5,7</sup>. It is not known whether the inhibition occurs directly at the cell surface or whether angiostatin is internalized by endothelial cells and subsequently inhibits cell proliferation. Both mechanisms could involve a receptor specifically expressed in proliferating endothelial cells<sup>3</sup>.

The *in vitro* culture of post-implantation rat embryos from 9.5 to 11.5 days is possible in homologous serum using the method described by New<sup>8</sup>, such that the development of embryos *in vitro* is comparable to that *in vivo*<sup>9</sup>. Despite numerous studies<sup>2,3,6</sup> have been carried out for explaining effects of angiostatin on cell culture there are no data about its embryotoxicity or teratogenicity. The aim of the present study was to investigate the *in vitro* effects of angiostatin (K1-3) on embryonic development during the organogenesis period in the rat.

## MATERIAL and METHODS

This study was approved by the Experimental Animals Ethics Committee of Erciyes University, Turkey. Wistar rats were obtained from the Clinical and Experimental Research Centre, Medical Faculty of Erciyes University. The female rats (approximately 8 weeks of age and weighing 150±175 g) were kept in cages in groups of five, and the males of the same strain were housed singly in mating cages. Male and female rats were placed together in the evening (one pair per cage), and the presence of a vaginal plug the following morning was taken to indicate mating had occurred.

On the assumption that mating occurred around midnight, the female was considered to be 0.5 day pregnant at noon the following day. The pregnant rats were killed by

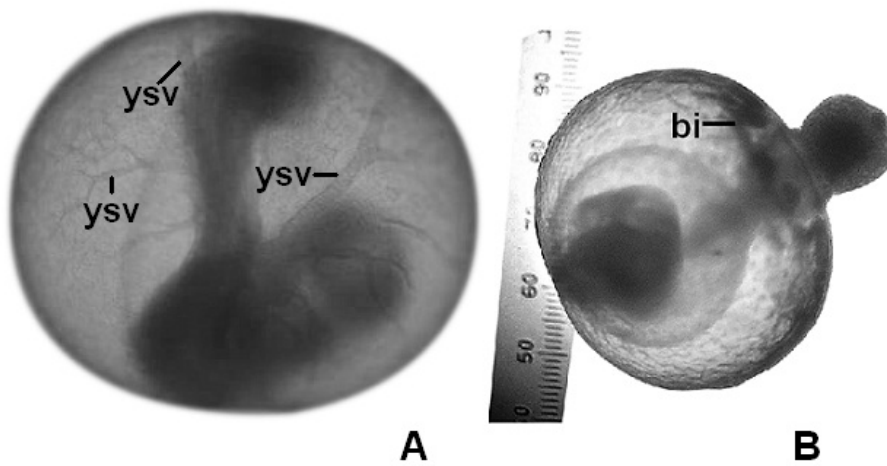
ether overdose at 9.5 days of gestation and the embryos (approximately 10 embryos from each pregnant rat) were removed from the mother by the explanation procedure described by New<sup>8</sup>.

In order to assess the effect of the angiostatin (K1-3) (TechnoGene, Israel) on total embryonic growth, embryos were divided in total 4 groups (10 embryos per one group) which were a control and 3 experimental. The control group embryos were cultured in whole rat serum (WRS). The administration dosages of angiostatin were determined according to the data gained from previous studies<sup>3-6</sup>. For the experimental groups, 0.5, 2.5, 5 µg/ml angiostatin were added to WRS. After 48 h culture the embryos from each group were examined under the dissecting microscope and assessed according to the morphological scoring system which takes account of the growth and differentiation of different embryological features, including the appearance of yolk sac circulation, allantois, body flexion, heart, caudal neural tube, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, branchial arches (bars), maxillary processes, mandibular processes, forelimbs, hindlimbs and somite number<sup>10</sup>. For all groups, protein contents of embryos were also determined with Folin Phenol reagents<sup>11</sup>.

Data of the morphological score and somite number, yolk sac diameter, crown-rump length and protein contents statistically were analysed. All datasets were subjected to normality test using the Kolmogorov-Smirnov method and the data were reported as either mean ± standard deviation ( $X \pm SD$ ) (for normally distributed data) or as median with 25%-75% percentile (for skewed data). Comparison of between the groups was made using the One Way Analysis of Variance (ANOVA; multiple comparisons were carried out with Tukey Test) or the Kruskal-Wallis Test (KW; Post-Hoc comparisons were carried out with Tukey Test). Statistical significance was set at  $P < 0.001$ . All analyses were performed with the statistical package for scientist (SIGMASTAT) Windows version 3.10.

## RESULTS

The embryos cultured in angiostatin (K1-3) showed severe growth retardation in all embryonic primordia when compared to embryos grown only in WRS (Fig. 1 and 2). Angiostatin affected the embryos in dose dependent manner and higher doses of angiostatin increased retardations of embryonic growth and development according to morphological scoring system and embryo protein content (Table 1). The lower morphological scores were accompanied by poor yolk sac vessel development according to the scoring system, some failure of fusion of the neural folds, incomplete embryonic flexion, and retarded development of otic, optic and olfactory systems, branchial bars, maxillary and mandibular processes and limbs. In addition to total morphological scores, yolk sac

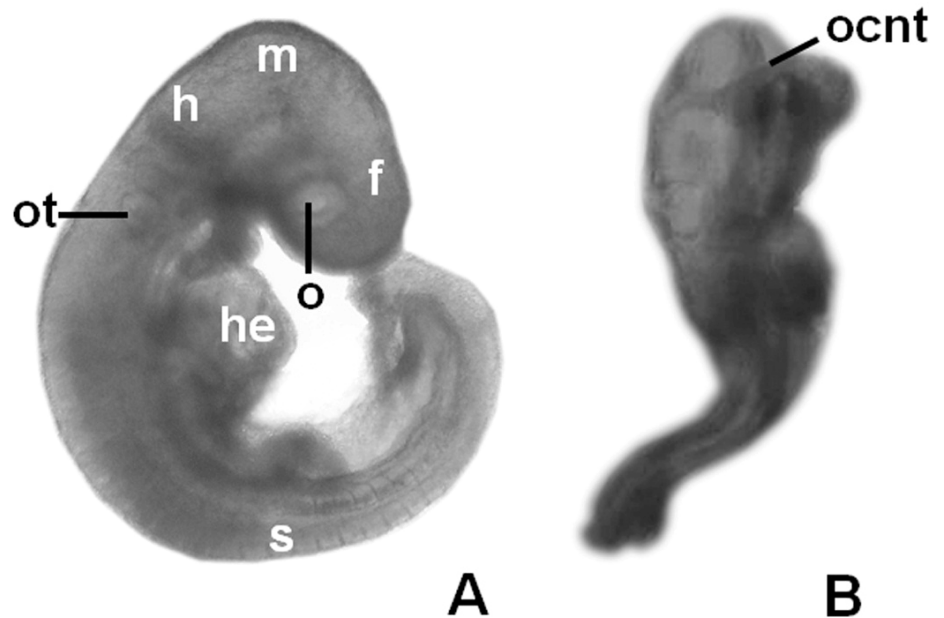


**Fig 1.** Rat embryos enclosed in the yolk sac at 11.5 days of gestation following 48-h culture period in WRS (A) and in WRS+5 µg/ml angiostatin (K1-3) (B). ysv, yolk sac vessel; bi, blood island

**Şekil 1.** WRS (A) ve WRS+5 µg/ml anjiostatin (K1-3)'de (B) 48 saatlik kültür periyodundan sonraki yolk kesesi içindeki 11.5 günlük rat embriyoları. ysv, yolk sac damarı; bi, kan adacı

**Fig 2.** Rat embryos outside of the yolk sac at 11.5 days of gestation following 48-h culture period in WRS (A) and in WRS + 5 µg/ml angiostatin (K1-3) (B). f, forebrain; m, midbrain; h, hindbrain; o, optic vesicle; ot, otic vesicle; he, heart; s, somite; ocnt, open cranial neural tube

**Şekil 2.** WRS (A) ve WRS + 5 µg/ml anjiostatin (K1-3)'de (B) 48 saatlik kültür periyodundan sonraki yolk kesesi dışına çıkarılmış 11.5 günlük rat embriyoları. f, ön beyin; m, orta beyin, h, arka beyin; o, optik vezikül; ot, otik vezikül; he, kalp; s, somit; ocnt, açık cranial neural tüp



**Table 1.** The statistical analyses in vitro embryonic development in whole rat serum (WRS), and angiostatin (K1-3)

**Tablo 1.** Total sıçan serumu (WRS) ve anjiostatin (K1-3)'deki in vitro embriyonik gelişimin istatistiksel analizi

Parameters		Control	0.5 µg/embr.	2.5 µg/embr.	5 µg/embr.	P
Total morphological score	X±SD	59.88±2.02 <sup>a</sup>	34.80±8.52 <sup>b</sup>	22.40±6.41 <sup>c</sup>	17.80±6.19 <sup>c</sup>	<0.001 <sup>§</sup>
Yolk sac diameter	X±SD	6.01±0.20 <sup>a</sup>	4.88±0.55 <sup>b</sup>	4.52±0.57 <sup>bc</sup>	4.22±0.39 <sup>c</sup>	<0.001 <sup>§</sup>
Crown-rump length	X±SD	5.41±0.23 <sup>a</sup>	4.46±0.42 <sup>b</sup>	4.18±0.87 <sup>bc</sup>	3.70±0.51 <sup>c</sup>	<0.001 <sup>§</sup>
Somite Number	Median (25%-75%)	24 <sup>a</sup> (24-25)	14 <sup>b</sup> (12-14)	12 <sup>b</sup> (11-17)	13 <sup>b</sup> (8-17)	<0.001 <sup>†</sup>
Embryo protein content	X±SD	140.76±12.21 <sup>a</sup>	112.66±8.67 <sup>b</sup>	89.85±10.89 <sup>c</sup>	53.77±8.15 <sup>d</sup>	<0.001 <sup>§</sup>

<sup>§</sup> One Way Analysis of Variance is applied

<sup>†</sup> Kruskal-Wallis test is applied

diameter, somite numbers and crown-rump length and embryo protein content of 11.5 days embryos grown in angiostatin were significantly decrease to normal embryonic development on day 11.5 in WRS.

Statistical studies showed that there was a significant decrease (P<0.001) total morphological score, yolk sac diameter, crown-rump length, somite number and embryo protein content. Mean morphological scores for the

embryos grown in WRS, in the presence of 0.5, 2.5 and 5 µg/ml angiostatin were  $59.8 \pm 2.02$ ,  $34.8 \pm 8.52$ ,  $22.4 \pm 6.41$  and  $17.8 \pm 6.19$  respectively. Yolk sac diameter was  $6.01 \pm 0.20$  mm in control group, and  $4.88 \pm 0.55$  mm,  $4.52 \pm 0.57$  mm and  $4.22 \pm 0.39$  mm in experimental groups respectively. While crown-rump length of control group was  $5.41 \pm 0.23$  mm, in experimental groups it diminished gradually due to the dose of angiostatin ( $4.46 \pm 0.42$ ,  $4.18 \pm 0.87$  mm and  $3.7 \pm 0.51$  mm). Median value of the somite numbers was 24 (24-25) in control group and it was diminished 14 (12-14), 12 (11-17) and 13 (8-17) respectively in experimental groups. The mean protein contents of embryos were also decreased in experimental groups ( $112.66 \pm 8.67$  µg/ml,  $89.85 \pm 10.89$  µg/ml,  $53.77 \pm 8.15$  µg/ml respectively) when compared to control group ( $140.76 \pm 12.21$  µg/ml).

While the yolk sac of embryos grown in WRS had a fully developed yolk sac plexus of vessels the yolk sac of embryos grown in WRS + 2.5 µg angiostatin had just established vitelline circulation and had few yolk sac vessels and WRS + 5 µg angiostatin groups had no vessels they had only blood islands (Fig. 1A and B). Developmental retardations in neural tube formation were also found in all experimental groups when compared to control group. There was open neural tube both cranial and caudal region in the embryos that grown in the presence of angiostatin (Fig. 2A and B).

Morphological analysis showed that all the embryos, grown in WRS + angiostatin, had open posterior neural tubes and less neural system development. In conclusion, angiostatin caused growth retardation in all experimental groups.

## DISCUSSION

Embryonic and adult growth processes are a prerequisite for the formation of a functional vascular system, which is essential for the proper development of vertebrate embryos, as well as for growth, regeneration and survival of adults<sup>12</sup>. The migration of endothelial precursors is one of the key mechanisms in the process of vascular development. When no preexisting vessels exist, they are derived from endothelial precursors<sup>13</sup>. The migration of endothelial precursor cells is regulated by various mechanisms and signals that initiate guide and stop the migration of endothelial precursors during formation of a vascular network. The vascular system originates from two fundamental processes: vasculogenesis and angiogenesis<sup>14</sup>. Vasculogenesis, the *de novo* formation of blood vessels from mesoderm, is driven by the recruitment of undifferentiated mesodermal cells to the endothelial lineage and their assembly into a primitive vascular network<sup>13</sup>. This process occurs mainly during fetal development, although recruitment into blood vessels of endothelial precursors, termed angioblasts, from bone marrow and peripheral blood have also been described

in adults<sup>15</sup>. The blood vascular system further extend by angiogenesis that corresponds to the generation of new blood vessels from endothelial cells of existing blood vessels, a process driven by endothelial cell proliferation and migration<sup>13</sup>.

Angiogenic process is a complex multistep cascade under the control of positive and negative soluble factors. The angiogenic switch is the result of the imbalance of positive and negative angiogenesis regulators as consequence of the up-regulation of endothelial growth factors or of the down regulation of natural angiogenesis inhibitors<sup>4,16,17</sup>. Our previous studies indicate that vascular endothelial growth factor<sup>18</sup>, platelet-derived growth factor<sup>19</sup> and basic fibroblast growth factor<sup>20</sup> could have important role on *in vitro* embryonic rat development because of their angiogenic effects. Up to now, because of the treatment of pathological angiogenesis, many physiologic angiogenic inhibitors have been characterized as angiostatin and endostatin<sup>2,21</sup>. Angiostatin is a proteolytic fragment of plasminogen that acts as an inhibitor of angiogenesis<sup>1,2</sup>. To evaluate the anti-angiogenic properties of angiostatin, many researches were made on *in vitro* angiogenesis models. *In vitro* endothelial cell culture models different concentrations of angiostatin (0.5-10 µg/ml) were used<sup>3,6,22-24</sup>. In addition to these studies, like above, different angiostatin concentrations (0.1-100 µg) were used on corneal neovascularization models by many researchers *in vivo*<sup>2,4,5</sup>.

Angiostatin treatment of endothelial cells in the absence of growth factors results in an increased apoptotic index whereas the proliferation index is unchanged. Angiostatin also inhibits migration and tube formation of endothelial cells<sup>23</sup>. Angiostatin binds to the  $\alpha/\beta$ -subunits of ATP synthase on the cell surface and this binding may mediate its antiangiogenic effects and the down-regulation of endothelial cell proliferation and migration<sup>25</sup>. In embryonic stem (ES) cell differentiation model system that angiostatin does not affect the steps of vasculogenesis involved in embryonic vascular development. The findings that angiostatin inhibits VEGF<sub>165</sub>-induced sprouting angiogenesis from embryoid body reflect that angiostatin may be a selective angiogenesis inhibitor. Elsewhere, in front of the early expression of its putative receptors, it remains to be established which cells are target cells of angiostatin during early developmental stages and whether angiostatin may affect cell compartment other than the endothelial one<sup>1,26</sup>. Alternatively, angiostatin may interact with an endothelial cell adhesion receptor such as integrin  $\alpha_v\beta_3$ , thus blocking integrin-mediated angiogenesis<sup>3</sup>. Indeed, several targets and various mechanisms of action have been proposed for the antiangiogenic action of angiostatin that remains to be fully elucidated.

In the present study, *in vitro* effects of angiostatin were tested on embryo culture. The results showed that

total embryonic growth was normal in WRS as opposed to the experimental groups, which were angiostatin was added in WRS, this figure decreased depending on the dose of angiostatin. Embryonic retardations were seen in total embryonic growth especially yolk sac diameter and vascularisation, crown-rump length, somite number, embryo protein content, body flexion and neural tube development. When postimplantation embryos were cultured in the presence of certain antiangiogenic molecules, similar regression was seen in embryonic growth, e.g. with anti-bFGF<sup>27</sup>, interleukin 12<sup>28</sup>. Our results indicated that the addition of higher concentrations of angiostatin resulted in growth retardation and some malformations. Those findings may be caused by the reduction of yolk sac functions, because the yolk sac decreased in size and vascularisation in the presence of these higher concentrations. It is well known that the yolk sac is an especially important placental organ in the rodentia at this time of embryonic development. It has been shown to be primary source of exchange between the embryo and mother during early embryogenesis before the chorioallantoic placenta becomes functional<sup>8</sup>.

Together with a wide range of other *in vitro* studies about the teratogenicity of several different molecules, the present findings suggest that the rat post-implantation embryo culture system is a very useful method for teratological studies and also particularly suitable for the assessment of specific effects on the morphogenetic events occurring during early organogenesis in mammalian embryos.

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