Original Article

The effect of kisspeptin-10 on angiogenesis and tumor growth

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Abstract

Background and Aim: Kisspeptins (kp) bind to a coupled-G protein receptor, GPR54 or KISS-1R, to perform a variety of functions including modulation of processes involving angiogenesis such as tumor metastasis and invasion of trophoblast. In this study the effects of kp-10, the most active kisspeptin, on angiogenesis and tumor growth have been evaluated.

Methods: Human umbilical vein endothelial cells (HUVECs) were treated with 10-100 or 500 nM kp-10 for 24 hours. Tube formation was evaluated in these groups and compared with nontreated cells with Matrigel-precoated 24-well culture dishes. In addition, treated adipose-derived mesenchymal stem cells with 10-100 or 500 nM kp-10 and nontreated cells were transfused via the tail vein to melanoma tumor bearing C57BL/6 mice. After 24 hours, the mice were scarified and number of vessels in tumor sections was evaluated by immunohistochemistry. Tumor size was measured with caliper and tumor volume was estimated. Kruskal-Wallis and Mann-Whitney tests were used to determine the difference between the treated and non-treated groups.

Results: The results showed that kp-10 significantly increased angiogenesis at 100 nM compared to the other groups both *in vitro* and *in vivo*. Moreover, a significant increase in the tumor growth was observed at 100 nM of kp-10 compared to other groups.

Conclusion: Our data demonstrated that kp-10 increases angiogenesis in tumor tissue, which may lead to increase in tumor size.

Key words: Angiogenesis, endothelial cell, kisspeptin-10, mesenchymal stem cell, tumor

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INTRODUCTION

Angiogenesis, the formation of new blood vessels from preexisting vessels, play an important role in physiological events such as growth and reproduction as well as pathological events including tumor growth and metastasis.[1] Angiogenesis is a complex process involving cell proliferation, migration, and vessel formation.[2,3] Special compounds (angiogenic factors) are released by tumor cells and stimulate formation of new blood vessels that plays an important role in the induction of tumor growth.[4,5] On the other hand, tumor cells release cytokines, chemokines, and growth factors that enable them to recruit and activate various nontransformed stromal cells such as mesenchymal stem cells (MSCs). MSCs are multipotent cells found in several adult tissues such as bone marrow and adipose tissue, which migrate to the sites of injury, ischemia and microenvironment.[5] In fact, growing tumors recruit MSCs in surrounding environment where they accelerate tumor growth in the primary sites and facilitate metastatic spread to the distant organs. Furthermore, it has been recently identified that MSCs can modulate tumor progression.[5] MSCs produce a number of growth factors influencing angiogenesis that facilitate growth of primary and metastatic tumors via induction of new blood vessel formation.[6]

One of the possible candidate suppressor genes that may play a major role in metastatic cascade is KISS-1 gene.[7] KISS-1 gene encodes a hydrophobic protein of 145 amino acid, which proteolytically can be cleaved to peptides known as kisspeptins (kp). These include kp-10, kp-13, kp-14 and kp-54, among which kp-10 is shortest and the

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most potent kisspeptin.[2,7] KISS-1 receptor activation by its ligands (kp) leads to regulating of various functions in both normal physiology and pathophysiology including angiogenesis and tumor metastasis.[8] Many studies have shown that loss of KISS-1 gene expression is related with progression of tumor in a large number of tumor types including malignant bladder[9], ovarian[10], gastric[11], and pancreatic[12] tumors.

Thus, this study aimed to investigate effect of kp-10 on angiogenesis that may affect the growth of tumor.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice 6-8 weeks of age were purchased from the Pasteur institute of Iran. The animals were used according to the guidelines of the Institute of Health for the care of laboratory animals and the ethics committee. This study was conducted in the Physiology Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran. The mice served as recipients for tumor inoculation in subcutaneous models of melanoma and injection of MSCs via the tail vein. The mice were divided into five groups and each one consisted of six mice. B16F10 melanoma cells (Cell bank of Pasteur Institute, Iran) were cultured in Dulbecco's Modified Eagle Media (DMEM, Sigma-Aldrich, USA) containing with 10% Fetal bovine serum (FBS, Invitrogen, USA), 1 g/L glucose, 1% L-glutamine and 1% penicillin-streptomycin at 37˚C in a CO_2 atmosphere in which 10^6 cells in 200 μl volume of phosphate buffered saline (PBS, Invitrogen, USA) were inoculated into the left back flank of the mice. Then, for each mouse about 5×10^5 MSCs after 24 hours of treatment with 10-100 or 500 nM of kp-10 (Anaspec, USA) and nontreated cells in 200 μl volume of PBS were injected into tail vein on day 21. The control group was injected with only melanoma cells. After 24 hours mice were scarified and number of blood vessel in tumor sections were evaluated by immunohistochemistry.[3,13-15]

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs, Cell bank of Pasteur Institute, Iran) were cultured in M199 (Gibco, USA) medium supplemented with 10% FBS, penicillin 100 IU/ml and streptomycin 100 μg/ml (Invitrogen, USA) and were incubated at 37˚C with 5% CO_2 and 95% O_2 concentration. Medium was changed every day and the third passage cells after reaching 80% confluency were used for experiments.[16]

Tube formation assay

Number of 1×10^5 HUVECs were treated with 10-100 or 500 nM of kp-10 (Anaspec, USA) for 24 hours, then this cells and nontreated cells were seeded into Matrigel-precoated 24-well culture dishes (Invitrogen, USA) for 24 hours at 37˚C. For evaluation of tube formation, cells were incubated with Calcein AM color (Sigma-Aldrich, USA) for 30 min in dark at 37˚C, then length, size and number of junctions were examined microscopically using a digital camera on a phase contrast microscope (IM50 Image manager; Leica). Photographs were obtained from wells and were assessed by Image-Pro 6.2 software program.[3]

Immunohistochemistry

For evaluation of angiogenesis, tumors were removed and fixed in formalin. Then 5 μm thick paraffin sections of tumor samples were incubated with anti-CD31 primary antibody (Ab) (eBioscience, USA) at concentration of 1:1000. Then these sections were incubated with a HRP-conjugated secondary Ab. Images were obtained by microscope attached with a camera and number of vessels were counted.[2]

Measurement of tumor growth

Mice were observed three times a week for evaluation of tumor growth, after which length and width of tumors were measured with caliper and volume of tumors was estimated with the formula, length $\times$ width^2 $\times$ 0.52 .[2]

Statistical analysis

All data were presented as mean ± SEM. Analysis of data was performed with SPSS 16.0 using the Kruskal-Wallis test followed by the Mann-Whitney test to determine the statistical difference at $P < 0.05$ between the control and experimental groups.

RESULTS

Results have demonstrated that kp-10 significantly increased vessel length, size and junctions in HUVECs at 100 nM compared with other groups, but there was no significant difference between the other groups ($P < 0.05$) [Figure 1].

Mean ± SEM of vessel length, size and junctions were as follows respectively in the nontreated cells; 20.1 ± 2, 55.8 ± 2, 0.69 ± 0.02, kp-10 (10 nM); 21.4 ± 2, 57.5 ± 2, 0.72 ± 0.02, kp-10 (100 nM); 45.9 ± 2, 132.1 ± 2, 2.43 ± 0.02, and kp-10 (500 nM); 20.4 ± 2, 57.4 ± 2, 0.75 ± 0.02.

The average vessels number at 100 nM of kp-10 was significantly increased compared to other groups, but the statistical significance was not observed between the other groups ($P < 0.05$) [Figure 2].

Mean ± SEM values were as follows in the Control; 2.8 ± 0.3, no treated cells; 3.5 ± 0.6, kp-10 (10 nM); 4.5 ± 0.6, kp-10 (100 nM); 8.6 ± 1.8 and kp-10 (500 nM); 4 ± 0.5.

Given that tumor growth is angiogenesis dependent, our results indicated that kp-10 significantly increased volume of tumors in the melanoma tumor bearing mice at 100 nM compared to other groups, but the significant difference was not observed between the other groups ($P > 0.05$) [Figure 3].

Results in mean ± SEM were as follows: Control; 1129.6 ± 410, no treated cells; 1228.6 ± 339.3, kp-10 (10 nM); 1686 ± 186, kp-10 (100 nM); 3287.3 ± 712.6, kp-10 (500 nM); 1442.5 ± 239.7.

DISCUSSION

In this study, we investigated the effects of kp-10 on angiogenesis and tumor growth and we showed that kp-10 at 100 nM significantly increased angiogenesis both *in vitro* [Figure 1] and *in vivo* [Figure 2] that may contribute to tumor growth [Figure 3].

Angiogenesis is a complex multi-step process that is essential for tumor growth. Many previous reports have

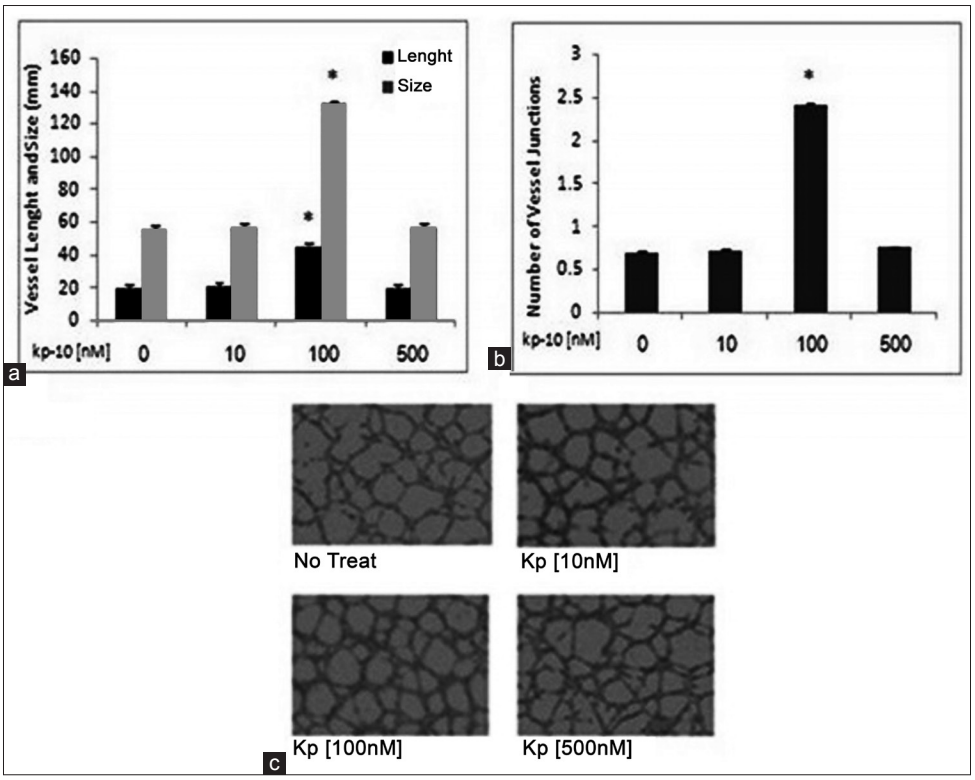

Figure 1: Effect of kp-10 on tube formation.
(a and b) Vessel length, size and junctions significantly increased at 100 nM of kp-10 compared to other groups, but no significant difference was observed between the other groups. (c) Microscopic images of tube formation in different concentrations of kp-10. Results are indicative two experiments and were compared between the groups with Kruskal-Wallis test (*P<0.05)

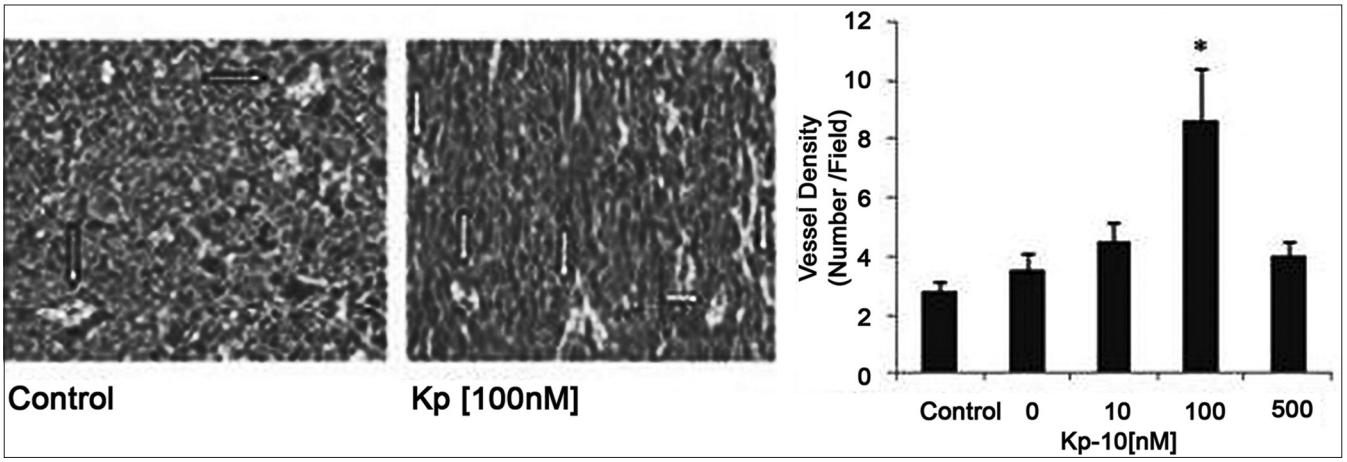

Figure 2: Effect of kp-10 on tumor angiogenesis in melanoma tumor bearing mice.
Arrows show cross section of capillaries lumen. The average vessels number was significantly increased at 100 nM of kp-10 compared to the other groups, but no significant difference was observed between the other groups. Results were compared between the groups with Kruskal-Wallis test (*P < 0.05)

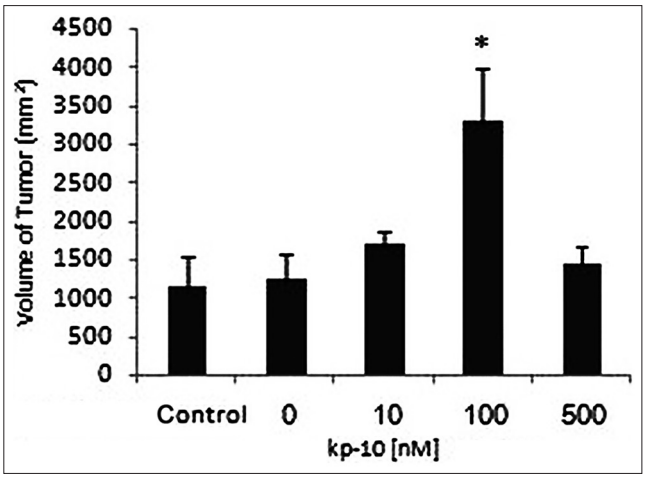

Figure 3: Effect of kp-10 on tumor growth.
kp-10 significantly increased volume of tumors at 100 nM compared to the other groups, but no significant difference was observed between the other groups. Results were compared between the groups with Kruskal-Wallis test (*P < 0.05)

shown that inhibition of tumor growth and metastasis is effective by blocking tumor angiogenesis.[2] KISS-1 gene was identified in human malignant melanoma cells as a metastasis suppressor gene, which is involved with each step of metastatic cascade including invasion, migration and angiogenesis. It has been shown that activation of GPR54 by kisspeptin inhibits motility and tumor cell invasion.[17] There is reliable evidence to suggest that kisspeptin can activate many different signals through GPR54, which include activation of phospholipase C, accumulation of inositol triphosphate and activation of protein kinase C. Kisspeptin also has active pathways that are associated with mitogen activated protein kinase (MAPK), particularly extracellular-signal-regulated kinases ½ (ERK1/2), p38, phosphatidylinositol 3′ kinase (PI3K) / AKT, matrix metalloproteinase (MMP) and interaction with chemokine receptor type 4 (CXCR4).[18]

Kisspeptin and KISS-1 receptor have been reported to be expressed in the aorta, coronary arteries, and human umbilical vein, which acts as a vasoconstrictor (a basic stage in angiogenesis) in isolated coronary arteries suggesting its role in the vascular system.[19]

Sawyer and colleagues showed that kp-10 is a vasoconstrictor in small peripheral vessels.[20] Some recent studies have shown that exogenous KISS-1/KISS-1R signaling can suppress tumor angiogenesis and distant metastasis.[8] Ramaesh, has shown that kp-10 at 1 nM to 1 μM concentrations significantly inhibits angiogenesis in placental vessels.[3] In another study it was demonstrated that kp-10 significantly inhibits tumor growth and angiogenesis at 10^{-1}–10 μM concentrations.[2]

So far, no study has examined the effect of kisspeptin on MSCs. MSCs can directly interact with a variety of different adjacent normal cell types. In case of tumor cells, such interactions with MSC would result in support of tumor-promoting activity.[21] Participation of MSCs in tumor development through increased angiogenesis with secretion of angiogenic factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and chemokine receptor type 12 (CXCL12) has been shown previously.[20]

MSCs were previously shown to enhance collateral remodeling of vessels, angiogenesis, and regeneration of tissues in several systems *in vivo* including assays in chick embryo chorioallantoic and in rat model for ischemia of hind limb or heart.[22,23] Suzuki and colleagues have suggested that injection of MSCs to melanoma tumor bearing mice promote tumor growth *in vitro* and *in vivo* that may be partly due to increased angiogenesis.[24] Also Kachgol reported that MSCs derived from different tissues can facilitate angiogenesis through distinct mechanisms.[25]

Since the migration and proliferation of cells are necessary for angiogenesis, mechanism to increase angiogenesis at 100 nM of kp-10 may be due to increased cell migration or proliferation. In support of this statement, we have shown in our other study that, kp-10 at 100 nM concentration increase proliferation as well as migration of MSCs and HUVECs (under publication).

Also, Zajac and colleagues have reported that kp-10 stimulated migration of MDA-MB231 and Hs578T breast cancer cells, with the maximum migration observed at 100 nM kp-10.[7] Therefore, it appears that there is a distinctive dose dependent pattern effect of kp-10 on angiogenesis, which may be exhibited through the distinct effects of different concentrations of kp-10 on signaling pathways such as MAPK, ERK1/2, AKT, and CXCR4. Furthermore, it has been suggested that KISS-1/KISS-1R signaling could be cell-type-specific.[8]

CONCLUSION

Our data showed that kp-10 at the concentration of 100 nM increases angiogenesis both *in vitro* and *in vivo* which could be responsible for the increased tumor growth.

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