RESEARCH ARTICLE

Lack of Effects of Recombinant Human Bone Morphogenetic Protein-2 on Angiogenesis in Oral Squamous Cell Carcinoma Induced in the Syrian hamster Cheek Pouch

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Abstract

Recombinant human bone morphogenetic protein-2 (rhBMP-2), a member of the TGF-β family, has been used widely in recent years to regenerate defects of the maxillary and mandible bones. Such defects are sometimes caused by resection of oral squamous cell carcinoma (OSCC) yet the biologic effects of rhBMP-2 on these carcinomas are not fully clear. The objective of this study was to determine histologically whether rhBMP-2 produces adverse effects on angiogenesis during induction of OSCC, a biologic process critical for tumor formation in an experimental model in the buccal pouch of golden Syrian hamsters. Buccal cavities were exposed to painting with 0.5% DMBA in liquid paraffin three times a week for 14 weeks, then biopsies were taken. Division was into 2 groups: a study group of 10 hamsters receiving 0.25μg/ml of rhBMP-2 in the 3rd and 6th weeks; and a control group of 10 hamsters which did not receive any additional treatment. VEGF expression and microvessel density were measured but no differences were noted between the two groups. According to this study , rh-BMP-2 does not stimulate angiogenesis during induction of OCSSs.

Keywords: Recombinant human bone morphogenetic protein-2 - oral squamous cell carcinoma - angiogenesis

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Introduction

Bone defects in the maxillofacial region vary from a small (few millimeters) periodontal defects to the large segmental defects resulting from trauma or surgical excision of malignancy. Such defects typically have complex three-dimensional structural needs, which are difficult to be restored by biomaterials alone without any osteoinduction (Hanasono, 2014; Sadigh et al., 2016).

The use of autogenous bone grafts has long been considered the golden standard of comparison for new bone graft materials (Cole et al., 1995). It has the advantages of being progressively incorporated into the bone tissue (osseointegrate), resisting infection, and being potentially capable of growth (osteogenesis) (Liao et al., 2016).

However, in the maxillofacial region, large defects that require bone reconstruction sometimes arise from resection of advanced malignancies. These defects requires a vast quantity of bone graft which is not easily afforded by autografts. In addition, autogenous bone graft transfer has many limitations, including donor site morbidity, difficulty restoring the complex 3-dimensional structure of the defect, significantly extending the surgical time, and a 5-10% failure rate due to vascular thrombosis (O'Malley et al., 2014; Buser et al., 2016).

These issues underscore the high interest in the search for an ideal alloplastic bone materials for reconstructing these defects and inducing the regenerative potential of the bone tissue itself.

Extensive preclinical studies in animal models have indicated to a successful healing rates of critical-sized bone defects using osteoinductive bone morphogenetic proteins (BMPs) delivered on biodegradable materials or by a gene therapy vectors (Hakki et al., 2014). The application of rhBMP-2 has been reported to have a good outcomes in normal bone induction at the site of implantation-a process includes the migration and proliferation of mesenchymal cells at the site of implantation- followed by their differentiation into osteoprogenitor cells (Marx et al., 2013; Gomes et al., 2016).

According to the success of prospective clinical trials, regenerative therapy using recombinant human BMP-2 (rhBMP-2) is FDA-approved since 2002 for fusion of the lumbar spine in skeletally mature patients with degenerative disc disease (DDD) at one level from L2-S1 and for healing of acute, open tibial shaft fractures stabilized with an IM nail and treated within 14 days of the

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initial injury. rhBMP-2 is also approved for certain oral and maxillofacial uses such as : alveolar ridge augmentation and sinus floor augmentation. However FDA requested the manufactures of biomaterials containing rhBMP-2 to include contraindications , including a history of cancer (Epstein, 2013).

The effects of BMP-2 on the malignant cells still controversial, many researchers have investigated the link between BMPs and cancer. many of them have pointed that several types of BMPs (such as BMP-2, BMP-4,BMP-6 and BMP-7) are implicated in many types of cancer tissues and also many studies reported their Dysregulation (Mancino et al., 2008; Le Page et al., 2009; Kim et al., 2015; Zaid et al., 2016). In spite of these extensive studies, the exact roles of the BMP signaling pathways in carcinogenesis remains poorly understood.

Angiogenesis is identified as: a process of new blood vessels formation from pre-existing vessels. It is a vital process in the growth and development, as well as in bone formation (Xie et al., 2016). However, it is also a fundamental step in the Tumor growth and metastasis (Jiang et al., 2008; Li et al., 2016).

Tumor angiogenesis, is a four-step process: first step is the local injury of the basement membrane, then endothelial cells is activated by angiogenic factors migrate. Third, endothelial cells proliferate and become stable. finally, angiogenic factors continue to influence the angiogenic process (Nishida et al., 2006).

There is a lot of angiogenic activators, including vascular endothelial growth factor (VEGF) which is considered a powerful angiogenic agent in neoplastic tissues. VEGF family express in cancerous tissues, and plays an important role in neovascularization Under the influence of certain cytokines and other growth factors (Hoeben et al., 2004).

Vascular endothelial growth factor (VEGF) is a potent regulator of angiogenesis and thereby involved in the development and progression of solid tumours (Luo et al., 2013). Vascular endothelial growth factor-A (VEGF-A) is a heparin-binding glycoprotein that occurs in at least six molecular isoforms , this factor is a potent and very specific mitogen for vascular endothelial cells and stimulates the full cascade of events required for angiogenesis (Bamberger and Perrett, 2002).

Langenfeld provided many evidences that BMP-2-induced angiogenesis occurs at least, in part, by stimulating endothelial cells. BMP-2 enhances tube formation, induces phosphorylation of Erk-1/2, Smad 1/5, and increases Id1 expression and that lead to activating endothelial cells (Langenfeld and Langenfeld, 2004). Other authors indicated that BMPs stimulate angiogenesis through the production of VEGF-A by osteoblasts (Deckers et al., 2002).

The biological effects of BMP-2 on oral squamous cell carcinoma (OSCC) must be well studied before using this protein for reconstructing defects caused by oral cancer. The purpose of this study was to determine histologically in vivo effects of rhBMP-2 on angiogenesis in OSCC tissues.

Materials and Methods

Animals

20 male golden Syrian hamsters, 8-10 weeks old, weighing 80-120 g, were maintained in the Animal Houses of the faculty of pharmacy, Damascus university, Syria. The animals were housed in polypropylene cages and were provided with standard pellet diet composed of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins, 55% nitrogen-free extract and water. The animals were maintained under controlled conditions of temperature (27±21C) and humidity (55±5%) with a 12 h light/dark cycle.

Materials

7,12-dimethylbenz[a]anthracene (DMBA) (95% purity, CAS NO.: 57-97-6 soluble in liquid paraffin) were purchased from Sigma Aldrich Chemical Pvt. Ltd. rhBMP-2 (0.25μg/ml, soluble in physiological serum) were purchased from Cowellmedi Co., Ltd Korea .

Tumor induction and experimental procedures

Hamster buccal pouch carcinogenesis was induced in the 20 golden Syrian hamsters by topical application of 0.5% DMBA in liquid paraffin three times a week for 14 weeks. rhBMP-2 (1μg / ml) was injected in the buccal pouch submucosal tissue of 10 animals (study group) at the 3rd and 6th weeks. Then animals were sacrificed and tissues samples were extracted and preserved in 10% buffered formalin.

Histopathological studies

Histopathological investigations were performed on buccal mucosa tissues of the control and experimental animals in each group. Tissues were fixed in 10% buffered formalin and routinely processed and embedded with paraffin; Two or three serial sections 4µm thick were prepared and placed on silanized slides. The sections were deparaffinized and rehydrated through xylene and descending grades of alcohol. Antigen retrieval was carried out in a pressure cooker in 10 mM citrate buffer

Figure 1. Hamster Buccal Pouch after 14 Days of DMBA application

(pH 6.0) for 2 to 5min. The sections were then incubated after covering them with 3% hydrogen peroxide for 15min to block any endogenous peroxidase activity, and then 20 slides incubated with primary antiVEGF-A Rabbit polyclonal antibody (Abcam Inc., Cambridge, MA) for 4h at room temperature using an optimal dilution of 6 μg/ml. After further incubation with the secondary antibody (45min) and streptavidin peroxidase (30min), visualization was performed using freshly prepared diaminobenzidine (DAB) chromogen for 10min. The slides were finally counterstained with Harris hematoxylin. Then the sections were examined under microscope to investigate the staining patterns and expression of VEGF-A in each group.

Five of the most invasive tumoral islands were captured and the whole epithelial cells in these islands were counted and examined. VEGF-A expression value was calculated by counting the percentage of the stained tumour or epithelial cells in addition to the expression degree. results were classified in three categories: *i*). Weak ± (less than 10% of the epithelial/tumour cells was positively stained and weak expression), *ii*). Moderate + (10% to 50% of the epithelial/tumour cells was positively stained and moderate expression), *iii*). Severe ++ (more than 50% of the epithelial/tumour cells was positively stained and severe expression)

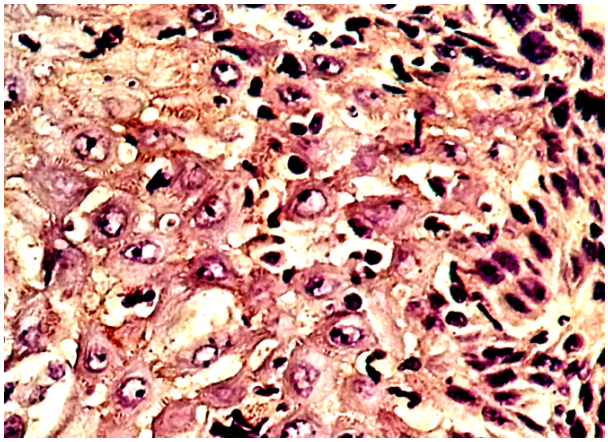

Figure 2. Expression of VEGF-A in Study Group

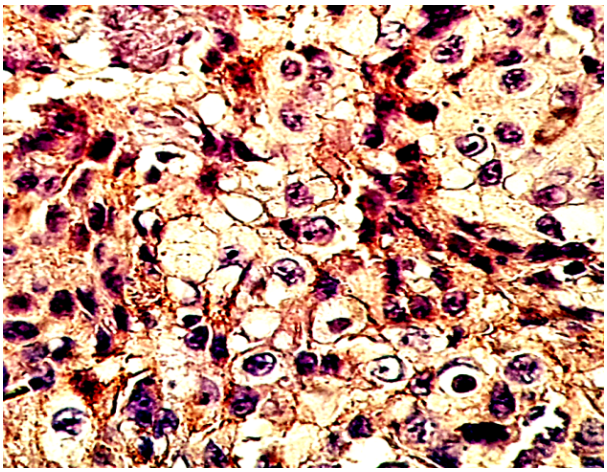

Figure 3. Expression of VEGF-A in control group

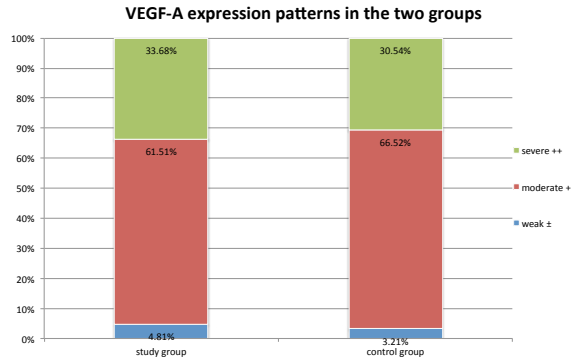

Figure 4. VEGF-A Expression Patterns in the Two Groups

Results

All of the tissue samples exhibited a positive expression patterns for VEGF-A . most of the samples showed moderate to severe staining as shown in Table (1) and Figure 2,3.

According to Kruskal-Wallis test there was no significant difference between the two groups (P>0.05). also, quantification of blood vessel density according to the immunohistochemical staining for VEGF-A factor showed that there were no significant changes between the OSCCs with and without treatment with rhBMP-2.

Discussion

Oral cancer is the sixth most common cancer worldwide, more than 90% of all oral cancers are squamous cell carcinomas (SCC) (Zaid, 2014), this type of cancers composes About 95% of oral cancers in India (Krishna et al., 2014).

Hamster buccal pouch carcinogenesis is an excellent model to study oral carcinogenesis because the development of 7,12-dimethylbenz[a]anthracene (DMBA)- induced squamous cell carcinoma in hamster buccal pouch simulates many of the histological, biochemical, and molecular alterations that occur in human oral carcinoma (Miyata et al., 2001) .

The regeneration of bone tissues requires the presence of the classic (tissue engineering triangle) which includes a source of cells, a signal for bone formation, and a matrix (Gomes et al., 2016).

Bone morphogenetic proteins are considered as part of a signal stimulator, large, multi-functional growth factor family with diverse effects on cell activity in development and regeneration. That is why several authors have

Table 1. Expression of VEGF-A in the Study

Group	median	Standard deviation	Highst value	Lower value
Study group	66.21%	0.31	88.10%	26%
Control group	64.33%	0.26	83.70%	29%

investigated the biological effects of Bone morphogenetic proteins on cancer tissues (Epstein, 2014).

The aim of this experimental study was to investigate the influence of rhBMP-2 on an essential biological process for tumor development which is angiogenesis . thus , the expression of vascular endothelial growth factor-A (VEGF-A) was investigated, this factor acts as an angiogenic factor and may play an important role In the cross-talk between endothelial cells and osteoblasts differentiation and induction with BMPs (Deckers et al., 2000), VEGF is a glycoprotein that acts as both a permeability and an angiogenic factor (Ferrara et al., 2003).

Determining the effects of rhBMP-2 on these tumor tissues in vivo is essential for determining whether these tissue engineering approaches could be used in patients with oral cancer or who had a history of OSCC.

Previous studies indicated that BMPs induces angiogenesis mediated by vascular endothelial growth factor A in the process of inducing osteoblast differentiation (Deckers et al., 2002). other studies have demonstrated that BMP-2 can stimulate angiogenesis in developing tumors (Langenfeld and Langenfeld, 2004; Raida et al., 2005), but in the present study no significant in vivo effects were found, and this result is completely in concordance with Gao et al who have found that rhBMP-2 does not produce any adverse effects on proliferation and angiogenesis in OSCC (Gao et al., 2010).

BMP-2 enhances tube formation, induces phosphorylation of Erk-1/2,Smad 1/5, during embryonic development(Lee et al., 2014) and increases Id1 expression and that lead to activating endothelial cells (Langenfeld and Langenfeld, 2004) . Targeted disruption of the BMP 2/4 transcription factor Smad 5 led to the disorganization of yolk sac vasculature and heart development (Jain et al., 2015). However, the role of BMP-2 on postnatal vascular development has not been clearly justified yet.

The demonstration that BMP-2 promotes tube formation, induces phosphorylation of p38, and increases Id1 expression shows that BMP-2 activates endothelial cells. BMP-2/4 has been shown to activate Erk-l/2 in osteoblasts (Lou et al., 2000) which in turn are located in proximity to endothelial cells. Vascular invasion is a prerequisite for endochondral bone formation after surgical resection of the malignant tissues as same as in fracture healing area (Bolander, 1992). Factors produced by endothelial cells may affect osteoblast function or differentiation and Osteoblasts are also able to produce paracrine factors that influence endothelial cell function, this cross-talk between the two types of cells, endothelial cells and osteoblasts which is mediated by VEGF-A may be the reason of the correlation found in many studies between BMP-2 and angiogenesis (Fiorelli et al., 1994; Carinci et al., 2005; Mattinzoli et al., 2016).

However , the results of this study and other studies need to be validated using human oral cancer cell lines in clinically relevant orthotopic animal models for oral squamous cell carcinomas.

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