Bone Morphogenetic Protein-6 Is a Marker of Serous Acinar Cell Differentiation in Normal and Neoplastic Human Salivary Gland

A. Kristiina Heikinheimo, Merja A. Laine, Olli V.-P. Ritvos, Raimo J. Voutilainen, Brigid L. M. Hogan, and Ilmo V. Leivo

ABSTRACT

Bone morphogenetic protein (BMP-6, also known as vegetal-pale-gene-related and decapentaplegic-vegetal-related) is a member of the transforming growth factor-β superfamily of multifunctional signaling molecules. BMP-6 appears to play various biological roles in developing tissues, including regulation of epithelial differentiation. To study the possible involvement of BMP-6 in normal and neoplastic human salivary glands, we compared its mRNA and protein expression in 4 fetal and 15 adult salivary glands and in 22 benign and 32 malignant salivary gland tumors. In situ hybridization and Northern blot analysis indicated that BMP-6 transcripts are expressed at low levels in acinar cells of adult submandibular glands but not in ductal or stromal cells. BMP-6 was immunolocalized specifically in serous acini of parotid and submandibular glands. None was found in primitive fetal acini or any other types of cell in adult salivary glands, including mucous acini and epithelial cells of intercalated, striated, and excretory ducts. All 16 cases of acinic cell carcinoma consistently exhibited cytoplasmic BMP-6 staining in the acinar tumor cells. Other cell types in these tumors, including intercalated duct-like cells, clear, vacuolated cells, and nonspecific glandular cells, exhibited no cytoplasmic BMP-6 staining. Other benign and malignant salivary gland tumors lacked BMP-6 immunoreactivity, except in areas of squamous differentiation. The results indicate that in salivary glands, BMP-6 expression is uniquely associated with acinar cell differentiation and suggest that BMP-6 may play a role in salivary gland function. More importantly, our experience of differential diagnostic problems related to salivary gland tumors suggests that the demonstration of consistent and specific BMP-6 immunoreactivity in acinic cell carcinoma is likely to be of clinical value.

INTRODUCTION

Tumors of the salivary glands account for about 5% of all neoplasms of the head and neck (1). These tumors originate primarily in the parotid gland, occasionally in the submandibular and intraoral glands, and rarely from the sublingual gland (2). The parotid gland is composed of serous acini, and the submandibular gland is composed of mixed serous and mucous acini. The sublingual and intraoral glands consist mainly of mucous acini. Salivary gland tumors present a histomorphological diagnostic challenge to clinical pathologists. They are characterized by substantial complexity of morphological features and overlapping histological patterns in different tumor entities. A further problem is a lack of markers applicable in diagnostic histopathology. Little is known about specific transcription and growth factors involved in human salivary gland tissue morphogenesis and cytodifferentiation (3–7). Identification of such molecules through basic research is likely to furnish potential new tools for improving molecular diagnostics in relation to salivary gland tumors. The TGF-β3 superfamily of >50 structurally related cytokines includes factors important in cell growth and differentiation (8, 9). Apart from TGF-β3, members of this multigene family include members of the activin/inhibin subunit gene family, members of the decapentaplegic-Vg-related gene family, including BMP-2, and the Müllerian inhibitory substance. TGF-β family members signal through heterodimeric complexes of type-I and type-II transmembrane serine/threonine kinase receptors (10). Downstream signaling is transduced by unique proteins of the Smad gene family (11). During organogenesis, TGF-β family members regulate cell growth and differentiation, and in adult tissues, they regulate regeneration and homeostasis (9). Some members of the TGF-β family act in a wide range of tissues. Others are restricted to specific cell types. For example, BMP-2 and BMP-4 are essential for early embryogenesis (12, 13). BMP-7 is required for eye and kidney development (14, 15), and growth and differentiation factor-9 is needed for ovarian function and female fertility (16).

BMP-6 (also known as Vgr-1 and DVR-6) is specifically expressed in suprabasal layers of the epidermis, central nervous system, and hypertrophic cartilage (17–19). In vitro, BMP-6 has been shown to inhibit cell division, to promote terminal epithelial differentiation, and to induce endochondral bone formation, osteoblastic differentiation, and neuronal maturation (19–22). Recent BMP-6 gene targeting experiments in mice have revealed no overt defects in tissues known to express BMP-6, suggesting that other BMPs may compensate for the loss of BMP-6 in such tissues (23). The overexpression of BMP-6 function has been implicated in relation to occurrence of psoriasis (24). In prostatic adenocarcinoma, induction of BMP-6 expression has been associated with tumorigenesis (25–28) and the formation of osteosclerotic deposits in metastatic progression (25, 28). BMP-6 mRNA has recently been shown to be expressed in a human salivary adenocarcinoma cell line (HSG-S8) but not in oral squamous cell, gastric, rectal, bladder, signet-ring cell, or thyroid carcinoma cell lines (29). Because little was known about the role of TGF-β family members in human salivary gland differentiation, we initiated a series of studies aimed at characterizing expression of TGF-β family members in developing and adult salivary glands and in salivary gland tumors. We report here that BMP-6 is uniquely expressed in normal and neoplastic acinar cells of serous phenotype, thereby providing a new molecular marker for use in diagnostic histopathology of acinic cell carcinoma.

MATERIALS AND METHODS

Fetal and Adult Salivary Glands. Four fetal and 15 adult salivary gland specimens were investigated (Table 1). Fetal submandibular gland tissues (15th to 20th gwk) were obtained in connection with legal abortions induced...
using prostaglandins. The study had been approved by the Ethical Committee of Helsinki Maternity Hospital, Finland. Normal adult salivary gland tissue samples were obtained in connection with surgery of the head and neck region after permission had been obtained from the Ethical Committee of Turku University Central Hospital, Finland. The patients were between 19 and 93 years of age and had received no long-term medication or radiotherapy before surgery.

**Salivary Gland Tumors.** Twenty-two benign and 32 malignant salivary gland tumors were studied. Tumor samples were obtained during surgical removal of tumors in Helsinki University Central Hospital, Finland and classified in accordance with the WHO classification of salivary gland tumors (2).

**Tissue-Sample Preparation.** Fresh, normal and neoplastic tissue samples were frozen promptly in liquid nitrogen and stored at −70°C until subjected to RNA analysis. For routine histology and immunohistochemistry, specimens were fixed in 10% neutral buffered formalin and embedded in paraffin.

### Table 1 Immunodetection of BMP-6 polypeptides in developing and adult normal human salivary glands with antibodies to a DVR-6 (18) to preorgan of murine BMP-6.

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### Table 2 Immunodetection of BMP-6 polypeptides in benign salivary gland tumors

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### Table 3 Immunodetection of BMP-6 polypeptides in malignant salivary gland tumors

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### Five-micrometer sections from each tumor were cut on aminolevulinic acid-treated glass slides (Sigma Chemical Co., St. Louis, MO) for immunostaining and histological staining with H&E. For in situ hybridization, cryostat sections were cut from two submandibular gland and two acinic cell carcinoma samples (Table 3, cases 1 and 3) and mounted on glass slides treated with a 2% solution of 3-aminopropyltriethoxysilane (Sigma; Ref. 30).

**Probes.** A sample of 242-bp human BMP-6 cDNA (31) in a pGEM-3 ribovector was kindly donated by Dr. Vicki Rosen (Genetics Institute, Cambridge, MA). For in situ hybridization, linearized plasmid templates were labeled with [35S]de-UTP (1000 Ci/mmol; Amersham, Aylesbury, Buckinghamshire, United Kingdom) by means of in vitro transcription using SP6 or T7 RNA polymerases (Promega Biotech, Madison, WI; Ref. 32). For Northern blot analysis, a 500-bp cDNA fragment was obtained by reverse transcription-PCR using 5’AC/GAG-AC/GG-AC/CTG-GTG-3’ and 5’G/G/CA/GCA-CT/TTT-GGA-GCC/GA/TA/3’ primers and U2OS-cell RNA-derived cDNA as a template. The cDNA was subcloned into a pGEM-T vector and verified by sequencing. A single-stranded antisense BMP-6 cDNA probe was generated by linear amplification as previously described (33) using the purified insert as a template and the above-mentioned antisense oligonucleotide as a primer. The β-actin cDNA was labeled with [32P]Pd-CTP (6000 Ci/mmol; Amersham) by means of random priming using an Oligolabeling Kit (Pharmacia LKB, Uppsala, Sweden).

**RNA Extraction and Northern Blot Analysis.** Total cellular RNA was extracted from eight normal (four submandibular, two parotid, and two sublingual) salivary glands, four benign salivary gland adenomas (three pleomorphic adenomas, one basal cell adenoma), and one acinic cell carcinoma as previously described (34). Poly(A)+ RNA was further extracted using the Poly(A) Track Kit (Promega) according to the manufacturer’s instructions. For control purposes, poly(A)+ RNA was extracted as above from HT1080 and…

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*F, female; Par, parotid gland; ++, all acinar tumor cells positive; M, male; Bmg, buccal minor gland; Lmg, labial minor gland; −, all cells negative; M, male; F, female.

*b All serous acini positive, all mucous acini negative, occasional ductal cells positive.

*c All serous acini positive, occasional ductal cells positive.

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**Table 3 Immunodetection of BMP-6 polypeptides in malignant salivary gland tumors**

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*All cells negative; M, male; F, female.

*b Weak extracellular staining in some stromal areas.

*c Areas of squamous metaplasia positive.

*d Occasional adluminal epithelial ductal tumor cells positive, surrounding myoepithelial tumor cells negative.

*e Stratified squamous tumor epithelium positive.

*f Squamous epithelium widely positive, no stratification.

*g Occasional well-differentiated squamous tumor cells positive.
U2OS cell lines as well as from fetal kidneys (16–17 gwk) and livers (15–16 gwk). For Northern blot analysis, poly(A) + RNA samples (3 µg) were run in a 1.5% agarose gel, transferred onto a nylon filter, and hybridized as previously described (34).

**In Situ Hybridization.** *In situ* hybridization was performed on cryostat sections as previously described (34). Briefly, before hybridization, frozen sections were fixed in 4% paraformaldehyde (PFA)/5 mM MgCl₂, rinsed in Tris-buffered saline, and treated with protease-K (0.5 µg/ml) for 5 min. The reaction was stopped by adding glycine (0.1 M in PBS), and the sections were postfixed in 4% PFA/5 mM MgCl₂, rinsed in 50% formamide/2× SSC at room temperature, and acetylated. The prehybridization and hybridization steps were performed according to Miettinen and Heikinheimo (34). After posthybridization washes and RNase A (Sigma) and T1 (Boehringer Mannheim, Mannheim, Germany) treatments, the sections were subjected to autoradiography by dipping the slides into film emulsion (NTB-2; Eastman Kodak Co., Rochester, NY) and exposed for up to 5 weeks at 4°C. After developing the slides (n-19 developer; Kodak), the sections were counterstained in Harris’ hematoxylin and assessed using dark- and light-field microscopy.

**Immunohistochemistry.** Affinity-purified polyclonal antibodies, α DVR-6, were used for immunolocamation of BMP-6 polypeptide on cryostat sections and formalin-fixed, paraffin-embedded tissue sections. The α DVR-6 antibodies were raised against the proregion of a mouse glutathione-S-transferase/BMP-6 fusion protein (18). The α DVR-6 antibodies are specific for human, mouse, and chicken DVR-6/BMP-6. Immunostaining was performed using a Vectastain Elite ABC staining kit (Vector Laboratories, Burlingame, CA), with diaminobenzidine tetrahydrochloride as the chromogen. Endogenous peroxidase was inactivated by treating the sections with 10% hydrogen peroxide in methanol for 5 min. To enhance the availability of the antigenic determinants, sections were pretreated using an antigen retrieval system (BioGenex Laboratories, San Ramon, CA). The sections were treated with 10% normal goat serum in PBS for 30 min at room temperature followed by the primary antibody, diluted 1:100 (α DVR-6) in 1% normal goat serum in PBS, and incubated overnight at +4°C. Sections were counterstained in Mayer’s hematoxylin and mounted with Permount (Fisher Chemicals, Fair Lawn, NJ). The specificity of the immunoreaction was monitored by replacing the primary antibodies with the corresponding nonimmune sera.

**RESULTS**

**BMP-6 mRNA in Normal and Neoplastic Salivary Glands.** Northern blot analysis revealed a single weak band of expected size (4 kb) for BMP-6 in two submandibular gland samples and in three tumor samples representing two pleomorphic adenomas and one basal cell adenoma (Fig. 1). Control mRNA from samples including HT1080 and U2OS cell lines and a human fetal kidney tested positive. Human fetal liver tested negative. *In situ* hybridization analysis detected low levels of BMP-6 mRNA in acinar cells of normal submandibular gland epithelium (Fig. 2, A and B). No BMP-6 transcripts were detected in ductal epithelial cells. The BMP-6 sense probe gave only a background signal (Fig. 2C). In acinic cell carcinoma, no BMP-6 hybridization signal above background level could be detected by *in situ* hybridization (data not shown).

**BMP-6 Polypeptides in Normal Salivary Glands.** Fetal submandibular glands, including primitive acini, tested negative (data not shown). In the parotid glands, prominent, uniformly positive cytoplasmic staining patterns were seen in all serous acini (Fig. 3A). Occasional BMP-6-immunoreactive cells were also seen in striated and excretory ducts of various sizes. Most ductal epithelial cells, however, tested negative. In submandibular (Fig. 3B) and intraoral minor glands, all serous acini or serous portions of mixed acini were consistently BMP-6-immunoreactive. All mucous acini and mucous portions of acini tested negative. Striated and excretory ducts of the...
BMP-6 in Salivary Glands and Their Tumors

BMP-6 Polypeptides in Malignant Salivary Gland Tumors. Expression of BMP-6 was studied in 12 cases of pleomorphic adenoma, most of which showed only little staining (Table 2). In two cases, BMP-6 was occasionally found in adluminal epithelial cells of ductal structures. In five cases, distinct positivity was noted in areas of squamous metaplasia (Fig. 3D). In three cases, extracellular staining was seen in some stromal areas, although no cellular source of BMP-6 could be detected in these cases.

Monomorphic adenomas studied included Warthin’s tumors and basal cell adenomas. Five cases of Warthin’s tumor were studied. No positivity was seen in the oncotic epithelium or lymphocytic stroma. In two of these tumors, however, small areas of squamous metaplasia were noted in the oncotic epithelium. These displayed positive staining for BMP-6 (Fig. 3E). We also studied five cases of basal cell adenoma and found no staining for BMP-6.

BMP-6 Polypeptides in Malignant Salivary Gland Tumors. Sixteen cases of acinic cell carcinoma were stained for BMP-6 (Table 3). They included five solid tumors, seven tumors with microcystic patterns of growth, three with papillary-cystic patterns, and one with a solid-microcystic pattern. All exhibited consistent strong cytoplasmic staining in acinar tumor cells (Fig. 4, A–D; Fig. 5, A and B). None of the other cell types in these tumors, including intercalated duct-like cells, clear, vacuolated cells, and nonspecific glandular cells were positive. In the papillary-cystic variant, only low amounts of positively stained acinar cells were detected among the nonspecific glandular cells and intercalated duct-like cells (Fig. 5, A and B). In any of our acinic cell carcinomas, there was no apparent correlation found between the intensity of lymphocytic inflammatory infiltrate typical of these tumors and amounts of BMP-6.

Four cases of adenoid cystic carcinoma were studied. They included two tumors with glandular (cribriform) patterns, one with a solid/glandular pattern, and one with a tubular pattern. No staining for BMP-6 was seen in the tumor cells of the two glandular (cribriform) cases (Fig. 4E). One case of tubular and one glandular (cribriform) type of adenoid cystic carcinoma exhibited weak positive staining in occasional adluminal epithelial cells; the surrounding myoepithelial cells were negative (Fig. 4F).

Ten cases of mucoepidermoid carcinoma were stained for BMP-6. Six exhibited no positivity for the protein. In two cases, stratified squamous carcinoma tumor cells were positive for BMP-6. In one other case, the squamous carcinoma tumor component was positive, although no stratification was found. In one case, individual well-differentiated squamous carcinoma cells occasionally tested positive (Fig. 4G). In all mucoepidermoid carcinomas, the mucin and mucous cell components and the intermediate cells consistently tested negative for BMP-6.

Two cases of intraoral polymorphous low-grade adenocarcinoma were studied. No positivity for BMP-6 was seen in either case. A consistently negative staining pattern was observed in all specimens studied when the primary antibodies were replaced by nonimmune control sera (Fig. 4D).

Discussion

BMP-6 was immunolocalized in this study, specifically in serous acini of human adult parotid and submandibular glands. Fetal acini and all other cell types in adult salivary glands, including mucous acini and epithelial cells of intercalated, striated, and excretory ducts, tested negative. The level of BMP-6 transcription in the acinar cells was, however, low, as measured by in situ hybridization and Northern blot analysis. Alternatively, the half-life of the transcript was short. Studies have shown that BMP-6 regulates growth and differentiation of epithelial tissue (18, 20, 24). In newborn mouse skin, BMP-6 was located in suprabasal but not in basal cell layers, suggesting that its expression is associated with terminal differentiation of keratinocytes.
In vitro, BMP-6 inhibited cell growth and induced keratinocyte differentiation, suggesting that BMP-6 function is a primary step in keratinocyte differentiation (20). It is concluded that in adult human salivary glands, BMP-6 expression is characteristic of serous acinar cells, which are highly specialized epithelial cells with a secretory function. BMP-6 originating in the saliva may be needed for the regeneration of oral mucous membranes, as demonstrated during keratinocyte differentiation (20).

The results of the work described here show that the 22 benign salivary gland tumors included in the study mostly lacked BMP-6 immunoreactivity. Occasional areas of squamous metaplasia in 5/12 of the pleomorphic adenomas and 2/5 of the Warthin’s tumors exhibited positive BMP-6 staining. This finding is in agreement with findings from previous work indicating a coexpression of BMP-6 with cytokeratin pair 1/10 of the suprabasal cytokeratins typical of cornifying stratified epithelia, e.g., in skin (20). Two cases of pleomorphic
adenoma and one case of basal cell adenoma expressed low levels of BMP-6 transcripts as detected by Northern blot analysis. The positive signal most likely originated from serous acini of the adjoining normal gland. Results of previous studies have suggested that BMP-2 in particular is responsible for the formation of the chondroid matrix in pleomorphic adenoma (29, 35–37). In the study described here, no BMP-6 immunoreactivity was detected in the chondroid areas of the pleomorphic adenomas, suggesting that BMP-6 expression in such tumors is associated more with epithelial than with chondroid differentiation.

In the various malignant salivary gland tumors we studied, all 16 cases of acinic cell carcinoma consistently exhibited distinct cytoplasmic BMP-6 staining in the acinar tumor cells. Other cell types, including intercalated duct-like cells, clear, vacuolated cells, and nonspecific glandular cells, tested negative. The level of BMP-6 transcription in the neoplastic acinar cells was, however, lower than in their normal cellular counterparts as measured by in situ hybridization and Northern blot analysis. Alternatively, BMP-6 mRNA degradation could be faster in the neoplastic acinar cells. The remaining malignant salivary gland tumors mostly lacked BMP-6 immunoreactivity, with the exception of 2/4 of the adenoid cystic carcinomas and 4/10 of the mucoepidermoid carcinomas in which occasional areas of BMP-6 immunoreactivity were detected in adluminal epithelial ductal tumor cells and in squamous tumor epithelium, respectively. Acinic cell carcinoma is the second most frequently encountered malignant epithelial tumor of the parotid gland after mucoepidermoid carcinoma. Histologically, acinic cell carcinoma displays cytological differentiation toward acinar cells. The parotid gland is the predominant site of occurrence (81% of cases). The results we report show that BMP-6 immunoreactivity, which is found in normal serous acinar cells, persists in the malignant counterparts of these cells, making BMP-6 a useful new marker of acinic cell carcinoma in diagnostic histopathology. In our experience of differential diagnostic problems relating to salivary gland tumors, this marker has already proved to be of clinical value.

Recent studies have shown that BMP-6 is expressed in human malignant prostatic tissues and in benign prostatic hyperplasia, especially in basal cells and areas of basal-cell hyperplasia, suggesting that BMP-6 may be involved in the differentiation of prostatic epithelial cells and in the genesis of prostatic hyperplasia and adenocarcinoma (25, 27, 28). It has been also suggested that BMP-6 function in malignant prostatic tissue may in part be responsible for the formation of calcified deposits in metastatic tumor sites in bone (25, 28). However, the findings in our study on acinic cell carcinoma show that BMP-6 expression need not have any association with hard-tissue formation or metastasis. It would be of interest to find out whether BMP-6 activity is related to the formation of the lymphatic infiltrate typical of acinic cell carcinoma in a manner analogous to the chemotactic attraction of monocytes by BMP-3 and BMP-4 (38).

Overall, we have shown that BMP-6 is uniquely associated with the serous acinar cell phenotype in normal and neoplastic human salivary glands. This shows that expression of certain members of the TGF-β superfamily may be characteristic of distinct cell types in both normal and neoplastic tissues. We conclude that BMP-6 is a useful new marker of acinic cell carcinoma in diagnostic histopathology.

ACKNOWLEDGMENTS

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