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ドライマウスの症状改善に対する高濃度水素水の有効例の検討

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はじめに

国内で800万人以上とも言われる口腔乾燥症は、高齢者を中心に増加している。原因は、シェーグレン症候群、老化、更年期障害、高血圧薬、抗アレルギー薬、向精神薬などの薬物、糖尿病、脳血管障害、ストレス、放射線治療、口呼吸、人工呼吸器の使用など多種多様であると言われている。このような外的因子により生体組織の障害を来たす原因として、強力な酸化力を有する活性酸素（フリーラジカル）が関与しているのではないかと言われている。口腔乾燥症もフリーラジカルにより口腔粘膜や唾液腺の萎縮・細胞死が進んだものとも考えられる。

口腔乾燥症に対する現治療法は、含嗽剤・保湿剤そして内服薬を用いているが、根治治療は困難で対症療法のみとなることが多い。そのため、安全かつ為害性がなく経済的にも負担とならない治療法の確立は有意義である。

最近、フリーラジカルを除去するスカベンジャーとして、抗酸化作用、認知性低下抑制などについての水素および水素水の有効性の報告が散見される[1,2]。そこで、口腔乾燥症に対する水素水の安全性と有効性に関する科学的検証をする目的で本研究を行った。今回はその研究中に著明な改善を示した症例を経験したので、その概要を報告する。

研究方法

研究は、臨床試験の計画を立て、水素水の安全性と有効性の科学的検証を行った。つまり、PhaseⅠでは、健常成人男性を対象に安全性の検討をした。前期PhaseⅡでは、口腔乾燥患者を対象として有効性、安全性及び有用性の検討を行った。有害事象の評価はCTCAE v.4にて行った。有効性を検討する評価のEndpointは、100mmVASスケールによる口腔乾燥感改善度とし、有効性の判定を行った。その他観察項目として、ガムテストによる唾液量、口腔内診査、血液・生化学検査の測定を行った。

本研究の研究施設は、国立病院機構栃木病院とし、前期PhaseⅡの被験者は当科を受診した患者で、対象となる口腔乾燥症の定義は、ガムテストで唾液量10cc以下、かつ口腔乾燥感の重症度VASスコア25mm以上とした。

なお、本研究は国立病院機構栃木病院の倫理委員会の許可を取得し、平成21年度厚労省科学研究・長寿科学総合研究事業の研究費により行った。

結　果

PhaseⅠ試験および前期PhaseⅡの結果は、平成21年度厚生労働省研究班報告書[3]において報告した。その報告では、全例grade２以下の有害事象であり、重篤なものは見られなかった。項目別では、頻尿が高頻度にみられたが、有意な用量反応性はみられなかった。頻尿に次いで、顔面および口唇の浮腫が多くみられた。これによる中断例はなかったが、数日間の飲用一時中止により改善した。以上のように、水素水によると思われる有害事象は認められ、飲用１日量は800ccまでとすることが推奨された。

また有効性においては、口腔乾燥症の重症度VASスコアの推移と安静時唾液分泌量の推移の評価項目において水素水の有効性が有意に認められた。また、１日量が800ccより1200ccの方が有効である傾向はあるものの、有意な用量依存はなかった。他覚的視診による口腔内の湿り気や粘膜炎に関する評価そして口腔内疼痛に関する自覚的所見による評価においても良好な改善がみられた。

以下、有効例4例を供覧する。

症例１

患　者　：　23歳、女性。

初　診　：　2009年12月。

主　訴　：　唾液が出ない。

現病歴　：　数年前より自覚するも放置していた。

現　症　：　口腔乾燥感VASスコア82mm、ガムテスト5cc／10min..
湿り気2、口腔粘膜炎0、疼痛0、ドライアイ（－）

既往歴　：　2008年8月よりアレルギー性皮膚炎にてステロイド軟膏（プロピオン酸クロベタゾール）を継続使用中。

経　過　：　一日の飲用水量を1200ccとして、水道水を2週間継続するも、唾液量の改善はなく、VASスコアも5mmの改善のみであった。
次に水道水を同量の水素水に変更した。唾液量、口腔乾燥の重症度VASスコア共に、飲用2週後には著明な改善を認めた（図９、10)。
有害事象は、Grade２の口唇浮腫とGrade１の頻尿がみられたが可逆的であり軽微であった。その他の事象としては、アレルギー性皮膚炎の改善がみられ、ステロイド軟膏の使用は必要なくなったとのことである。

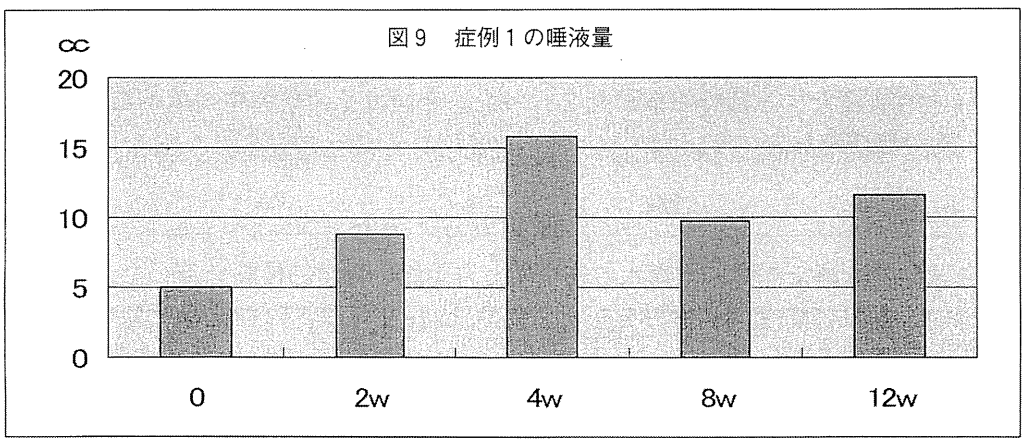


図９　症例１の唾液量

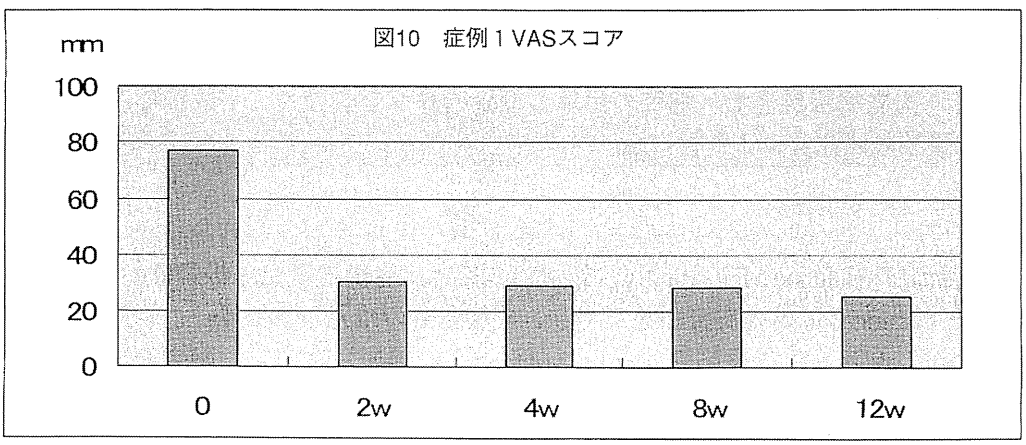


図10　症例１VASスコア

症例2

患　者　：　79歳、女性。

初　診　：　2007年5月。

主　訴　：　下顎歯肉およびオトガイ部の腫脹。

現病歴　：　悪性リンパ腫にて、2007年7・8月化学療法（エンドキサン、プレドニン、塩酸プロカルバシン、オンコビン）、2007年9・10月放射線（Liniac40Gy）施行。その後より口腔乾燥を自覚。

2007年11月～2008年1月まで口腔乾燥治療薬サラジェンを投与するも、唾液量の改善なく、また多汗のため中止となった。以降は含嗽剤のみとなった。

現　症　：　口腔乾燥感VASスコア58mm、ガムテスト2cc／10min.

湿り気2、口腔粘膜炎1、疼痛0、ドライアイ（－）

既往歴　：　特記事項なし。

経　過　：　一日の飲用水量を800ccとして、水道水を2週間継続するも、唾液量、VASスコア共に改善はなかった。

次に水道水を同量の水素水に変更した。水素水飲用8週後には著明な唾液量の増加を認め、口腔乾燥の重症度VASスコアも唾液量と関連して改善した（図11、12）。

有害事象は、Grade 1 の多汗、Grade 1 の口唇浮腫、Grade 1 の頻尿といずれも軽微であった。

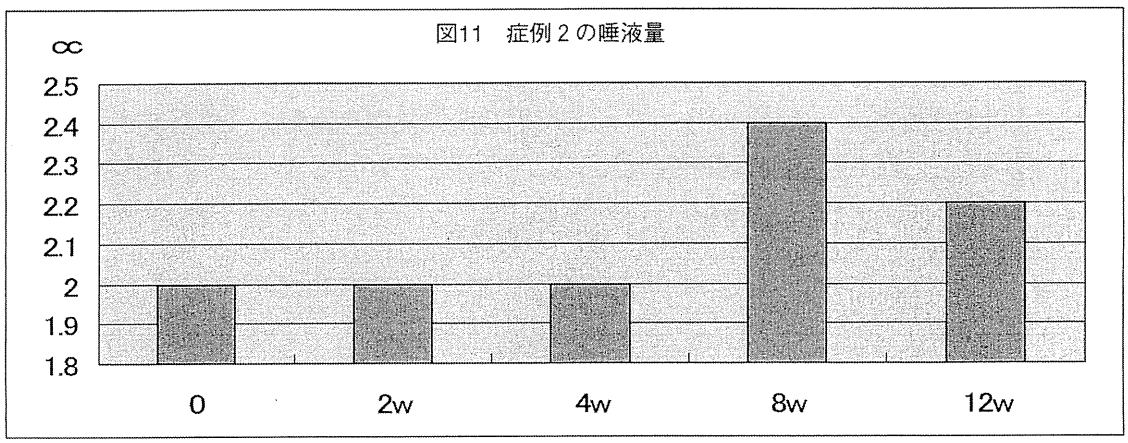


図11　症例2の唾液量

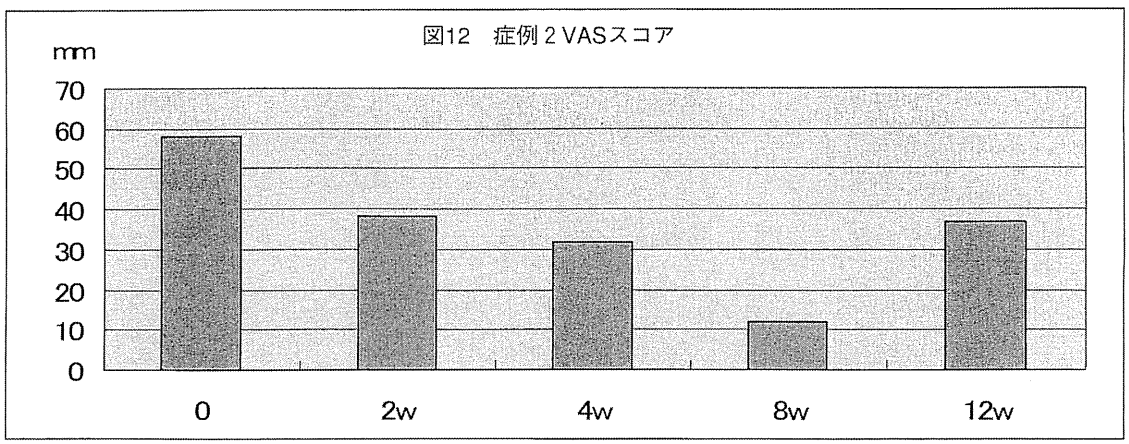
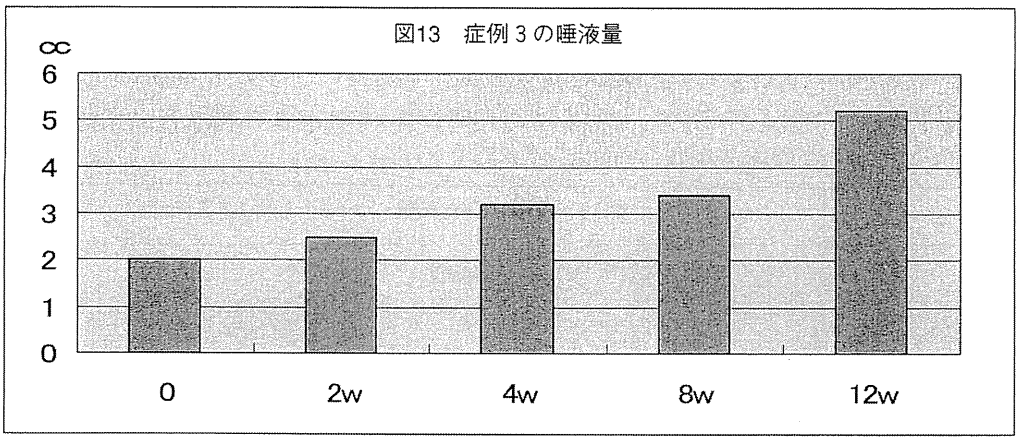
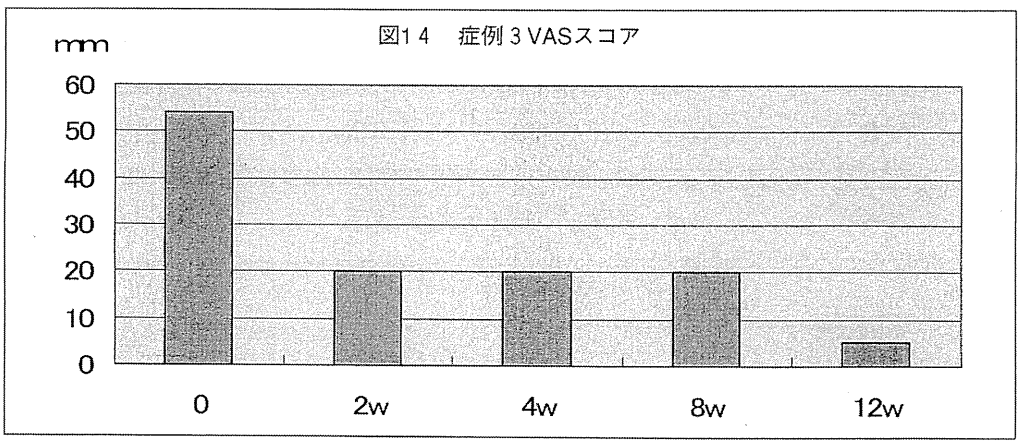


図12　症例2 VASスコア

症例３
患　者　：　92歳、女性。
初　診　：　2009年12月。
主　訴　：　口腔乾燥および舌痛。
現病歴　：　舌扁平苔癬にて含嗽剤とステロイド軟膏のみ。
現　症　：　口腔乾燥感VASスコア54mm、ガムテスト2cc／10min.
湿り気2、口腔粘膜炎2、疼痛1、ドライアイ（－）
既往歴　：　特記事項なし。
経　過　：　一日の飲用水量を800ccとして、水道水を2週間継続するも、唾液量、VASスコア共に改善はなかった。
次に水道水を同量の水素水に変更した。水素水飲用後には、唾液量は漸次増加を認め、口腔乾燥の重症度VASスコアは比較的早期に改善した（図13、14）。
有害事象は、Grade１の頻尿のみであった。





症例4

患　者　：　84歳、女性。

初　診　：　2005年10月。

主　訴　：　右上歯肉腫瘤。

現病歴　：　右上歯肉癌（T2N2aM0,SCC）にて、2005年11・12月放射線Liniac40Gy＋抗がん剤（CDGP+5FU）治療をし、2005年12月上顎骨部分切除術および頚部廓清術施行。2006年1月～12月まで抗がん剤（UFT300mg）内服をし、現在局所再発および遠隔転移なく経過良好。
一次治療後より口腔乾燥を自覚。2006年4月～2009年9月まで、口腔乾燥治療薬サラジェン15mgを内服するも口腔乾燥感および唾液量の改善はなかった。

現　症　：　口腔乾燥感VASスコア60mm、ガムテスト2.4cc／10min.
湿り気2、口腔粘膜炎1、疼痛1、ドライアイ（－）

既往歴　：　うつ病にて2007年12月～アンプリット10mg＋メイラックス1mg内服中。

経　過　：　一日の飲用水量を1200ccとして、水道水を2週間継続するも、唾液量、VASスコア共に改善はなかった。
次に水道水を同量の水素水に変更した。唾液量飲用2週後には著明な改善を認めた、口腔乾燥の重症度VASスコアも漸次改善し12週後には0となった（図15、16）。
有害事象は、Grade 1 の多汗、Grade 1 の口唇浮腫、Grade 1 の頻尿およびGrade 2 の膵炎がみられた。膵炎との因果関係は明らかではなかった。

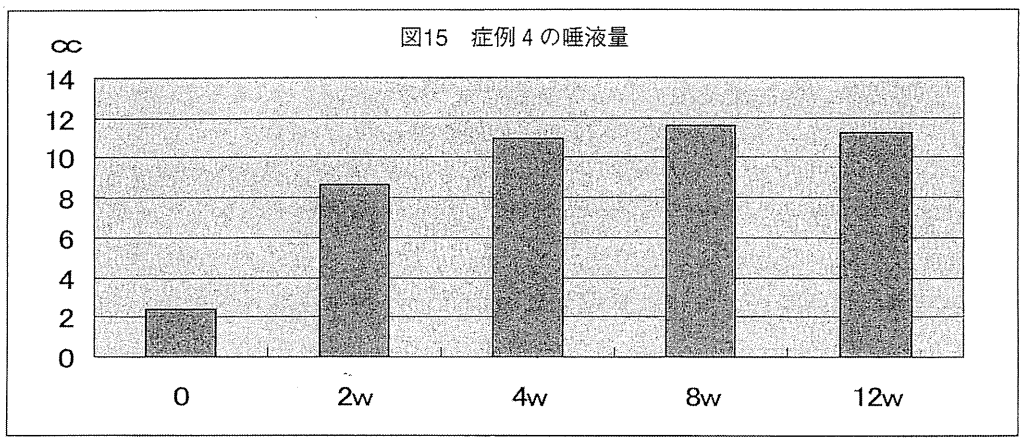


図15　症例4の唾液量

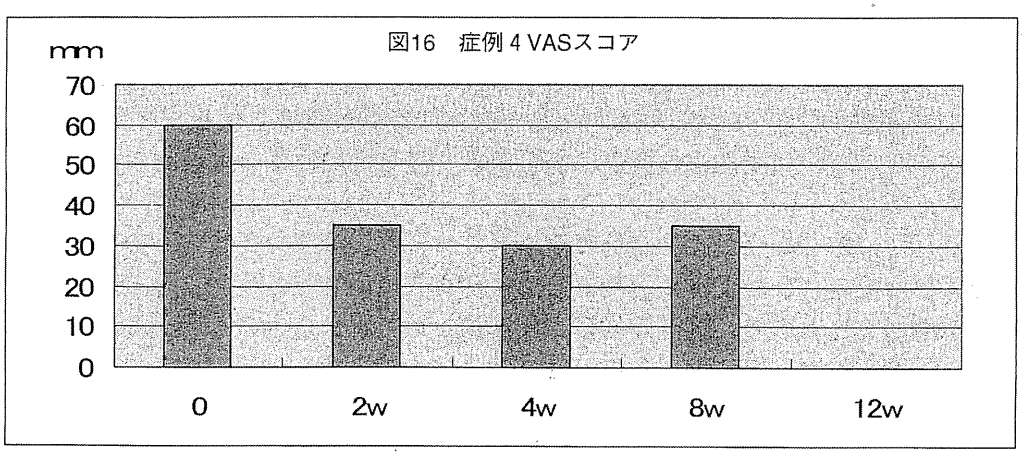


図16　症例4 VASスコア

考　察

国内で800万人以上とも言われる口腔乾燥症は、高齢者を中心に増加している。原因は、シェーグレン症候群、老化、更年期障害、高血圧薬、抗アレルギー薬、向精神薬などの薬物、糖尿病、脳血管障害、ストレス、放射線治療、口呼吸、人工呼吸器の使用など多種多様であると言われている。原因のいかんに係わらず、それらにより強力な酸化力を有する活性酸素（フリーラジカル）が産生され、これにより口腔粘膜や唾液腺の萎縮・細胞死が進んだものとも考えられる。

現治療は、含嗽剤・保湿剤そして内服薬を用いているが、根治治療は困難で対症療法のみとなることが多い。そのため、安全かつ為害性がなく経済的に負担とならない治療法の確立は有意義である。最近、フリーラジカルを除去するスカベンジャーとして、抗酸化作用、認知性低下抑制などについての水素および水素水の有効性の報告が散見される。この度、平成21年度厚労省科学研究で、口腔乾燥症に対する水素水の安全性と有効性に関する科学的検証をする機会を得、研究を行った。

活性酸素は生命を維持するために無くてはならないものである。酸素は細胞のミトコンドリアで糖質から電子を奪いスーパーオキシド→過酸化水素→ヒドロキシラジカルを経て水になる。この反応中に酸素は何度か活性酸素に変わるが、全ての活性酸素が水になる訳ではなく、余った活性酸素は細胞に損傷を与えると言われている。

細胞内の酵素で分解しきれない余分な活性酸素は、生体内において非特異的な化学反応を起こし、癌や生活習慣病そして老化等さまざまな病気の原因の一つとして、その有害性（酸化力）が指摘されている。この内、強い酸化力を有するスーパーオキシドアニオンラジカルやヒドロキシルラジカルをフリーラジカルと呼んでいる。

フリーラジカル発生因子としては、紫外線、電磁波、アルコール、煙草、筋肉疲労、過呼吸、過酸化脂質、化学物質、有害ガス、食品添加物、ストレスなどが挙げられる。

活性酸素（フリーラジカル）を消去する作用を有する物質をスカベンジャーと言い、体内各組織にはカタラーゼ、スーパーオキシドディスムターゼ、ペルオキシダーゼなどの抗酸化酵素が存在し、食物中のビタミン、カロチン、カタラーゼ等がスカベンジャーとして作用している。しかし無害化されずに残ってしまう活性酸素もあり、これが生体内で問題となる。 特に、最も強力な酸化作用を有する活性酸素のヒドロキシラジカルにはスカベンジャーがないと言われ、水素はこれに特異的に作用すると言われている。

活性酸素（フリーラジカル）の関与が考えられている疾患として、脳梗塞、老化、認知症、心筋梗塞、糖尿病、膀胱炎、アトピー性皮膚炎、リウマチなどが言われている。これらに対しての水素の有効性に関しての基礎的あるいは臨床的研究報告も散見される。その点から考え、ドライマウスにもフリーラジカルが関与しているのではと推測し本研究を計画した。

水素水の安全性については、高頻度に発現した有害事象として「頻尿」があった。供覧した全症例にもGrade 1 の頻尿がみられた。これは水素水の中断により改善したことより「明らかに関連がある」と考える。日常生活に支障は出なかったが、頻尿に伴う全身への影響は危惧される。また、これに関しては、用量依存性はなかった。この利尿作用の機序は不明であり安全性の面からも検討の必要性はあると考える。

次いで多く発現したのが「顔面および口唇の浮腫」であった。治療を要するものではなかったが、供覧症例１，２および４でみられたように、唾液分泌量が著明に増加した症例にみられ、口腔内の唾液腺が反応し分泌量が増加したものの、排泄障害により浮腫として現れたものと考える。水素水の飲用一時中断により軽快したことより、「明らかに関連がある」と考えられた。これに関しても用量依存性ではなかった。

重篤なものとして、症例４にみられた急性膵炎があった。１日量1200ccの水素水を飲用して8週後に発現した。膵炎患者は、その治療のために経口制限があった時期を除き水素水を継続したが、その後再発・再燃はなく経過良好となっている。同症例は唾液分泌量の著明な改善がみられたこと、発症時には水素水以外は向精神薬のみの内服だけであったことより、膵炎と水素水とには「関連あるかもしれない」と思われる。

水素水の有効性については、供覧した全ての症例においてもみられたが、口腔乾燥症の重症度VASスコアと安静時唾液分泌量は有意に改善した。また、他覚的視診による口腔内の湿り気や粘膜炎に関する評価そして口腔内疼痛に関する自覚的所見による評価においても良好な改善がみられた。１日用量に関しては、１日量が800ccより1200ccの方が有効である傾向はあるものの、有意な用量依存はなかった。

口腔乾燥症の重症度VASスコアの著明な改善は、安静時唾液分泌量増加率の改善により、口腔内の湿り気が出て、口腔粘膜炎の消失とそれに伴っての疼痛の消失によるものと思われる。口腔粘膜炎は、唾液分泌量増加率の改善よりも比較的早期にみられる傾向があり、水素水のスカベンジャーとしての効果あるいは抗炎症効果があったのではないかと思われた。

口腔乾燥症のみならず、口腔領域における水素の有効性に関する報告は未だない。本研究により、水素の効果が科学的に検証されれば、口腔乾燥症の治療法の１つとなるばかりか、広く口腔内病変への応用も期待されると考える。

平成21年度厚労省科学研究の報告書において、「老年病対策としての高濃度水素水による口腔乾燥症（ドライマウス）の症状改善に対する科学的検証～Phase Ⅰ及び前期PhaseⅡ臨床試験～」というタイトルで本研究のデータの一部を報告しているが、中年期の口腔乾燥症が多いことより対象を拡大し、さらに有害事象の評価は本年度よりCTCAE v.4に変更されたため、以前のデータも評価し直した。そのため今回は、既に報告した論文の症例に追加し、解析も新たに行い、改めて報告した。

謝　辞

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Dual effects of heparin on BMP-2-induced osteogenic activity in MC3T3-E1 cells

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Abstract:
Heparin displays several types of biological activities by binding to various extracellular molecules, including pivotal roles in bone metabolism. We have previously reported that heparin competitively inhibits the binding activity of bone morphogenic protein-2 (BMP-2) to BMP and the BMP receptor (BMPR) and suppresses BMP-2 osteogenic activity. In the present study, we examined whether heparin affects osteoblast differentiation induced by BMP-2 at various time points *in vitro*. We found that 72 h of treatment with heparin inhibited alkaline phosphatase (ALP) activity. However, 144 h of treatment enhanced the ALP activity in BMP-2-stimulated MC3T3-E1 cells. Although heparin decreased the phosphorylation of Smad1/5/8 after 0.5 h of culture, prolonged periods of culture with heparin enhanced the Smad phosphorylation. In addition, 72 h of treatment with heparin enhanced the mRNA expression of runx2 and osterix in BMP-2-stimulated MC3T3-E1 cells. Furthermore, the mRNA expression of BMP antagonists and inhibitory Smads induced by BMP-2 was preferentially blocked by heparin at the 24 and 48 h time points. These findings indicate biphasic effects of heparin on BMP-2 activity and suggest that heparin has complex effects on the BMP-2 osteogenic bioactivities. Prolonged culture with heparin stimulated BMP-2-induced osteogenic activity *via* down-regulation of BMP-2 antagonists and inhibitory Smads.

Key words:
osteoblast, heparin, BMP-2, Smad, Runx2, osterix

Abbreviations: ALP – alkaline phosphatase, BMP – bone morphogenic protein, BMPR – BMP receptor, ECM – extracellular matrix, GAG – glycosaminoglycan

Introduction

The extracellular matrix (ECM) provides structural strength to tissues and helps to maintain the shape of organs. Proteoglycans, which are characterized by a core protein with at least one glycosaminoglycan (GAG) chain attached, commonly mediate the interactions of ECM components with extracellular molecules, including growth factors, adhesion molecules, and cytokines. Recently, the potential roles of GAGs in various biological processes [27, 30], including angiogenesis [26], viral invasion [29], tumor growth [34], and bone metabolism [1, 3, 28], have been reported.

Well-known endogenous GAGs include heparin, heparan sulfate, keratan sulfate, chondroitin sulfate and hyaluronic acid. GAG structures are based on a disaccharide repeat. Four classes of GAGs exist and

are each distinguished by a particular repeating disaccharide. Among them, heparin is based on a repeat disaccharide of iduronic acid-(β1-4)-*N*-acetylglucosamine-(α1-4).

Bone morphogenic proteins (BMPs) were originally identified as unique proteins in demineralized bone matrix that induce ectopic bone formation upon implantation into muscular tissues [33]. BMPs were later shown to regulate the differentiation and function of cells that are involved in bone and cartilage formation and degradation, including osteoblasts, chondrocytes, and osteoclasts [4].

Signaling through BMPs is initiated by binding to the specific transmembrane receptors, type I and type II serine/threonine kinase receptors [37]. Type I receptors are activated by ligand bound-type II receptors and then phosphorylate downstream molecules in the cytoplasm. Further, Smad 1/5/8 transcription factors are substrates that are phosphorylated by the BMP receptor (BMPR) in the cytoplasm and accumulate in the nucleus within 1 h after BMP stimulation [35]. Phosphorylated Smads directly regulate the expression of primary target genes by binding to their promoter or enhancer elements together with Smad 4 and other transcription factors [12].

Recently, we have found that heparin inhibits BMP-2 osteogenic bioactivity by binding to both BMP-2 and BMPR. However, the effects of GAGs, including heparin, on BMP activity have not been fully examined. For example, heparan sulfate/heparin chains have been found to bind to BMP-4 and restrict the expression pattern of BMP-4 in Xenopus embryos [22]. Heparan sulfate also binds to noggin, a secreted polypeptide that inhibits the function of BMP, resulting in modification of BMP-4 activity [23], while heparan sulfate chains bind to BMP-7 and the heparan sulfate/BMP-7 interaction is required for BMP-7 signaling [11]. In addition, heparan sulfate and heparin inhibit BMP-2 osteogenic activity by sequestering BMP-2 on the cell surface and mediating the internalization of BMP-2 [14]. In contrast, some studies have reported that heparin enhances the biological activities of BMP-2 by protecting BMP-2 from degradation and inhibition by BMP antagonists [32, 38]. Thus, the mechanism by which heparin regulates bone metabolism induced by BMP-2 remains unclear. We hypothesized that heparin can act as either a negative or positive modulator of BMP activity depending on its action time. Because we have already reported that heparin suppresses BMP-2-induced osteogenic activity [16], we examined the effects of heparin on osteoblast differentiation induced by BMP-2 for prolonged periods of time in the present study.

Materials and Methods

Reagents

Porcine intestinal mucosal heparin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human BMP-2 was kindly supplied by Astellas Pharmaceutical Inc. (Tokyo, Japan). The anti-phospho Smad 1/5/8 polyclonal antibody, anti-phospho-p38 MAPK polyclonal antibody, and anti-p38 MAPK polyclonal antibody were obtained from Cell Signaling Technology, Inc. (Beverly, CA, USA). The anti-Smad 1/5/8/9 polyclonal antibody was purchased from Abcam (Cambridge, UK).

Cell culture

MC3T3-E1 cells, an osteoblastic cell line established from mouse calvaria, were cultured in α-minimum essential medium (α-MEM; Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; Gibco), penicillin G (100 U/ml), and streptomycin (100 μg/ml). The cells were maintained at 37°C in an atmosphere containing 5% CO_2.

Alkaline phosphatase (ALP) activity

Quantitative analysis of ALP activity was performed biochemically using the Bessey-Lowry enzymological method [2]. Cells were distributed in 24-well plates at a density of 1×10^5/well and incubated for 24 h. The growth medium was changed, and the cells were cultured with or without BMP-2 (100 ng/ml) and heparin (100 μg/ml). After an additional 48–144 h of incubation, the cells were washed twice with Hank's balanced salt solution (HBSS) and solubilized with HBSS containing 0.2% Nonidet P-40. The ALP activity of the lysate was determined using *p*-nitrophenylphosphate (pNPP; Wako, Osaka, Japan). After a 30-min incubation at 37°C, the absorbance of pNPP was measured at 405 nm using a Multiscan JX microplate reader (Thermo Fisher Scientific, Rockford, IL, USA). The ALP activity was normalized for protein concentration using the DC protein assay kit (Bio-

Rad, Hercules, CA, USA) measured by spectrophotometry at 630 nm. The specific activity of alkaline phosphatase was calculated as μM/μg protein.

RT-PCR analysis

Gene expression levels were determined using a reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA was extracted using a Total RNA Extraction Miniprep System (Viogene Co, Sunnyvale, CA, USA) according to the manufacturer's instructions, and the reverse transcript was subjected to PCR. Oligonucleotide primers were designed to amplify cDNA fragments encoding Runx2 (381 bp) and osterix (497 bp). The following primers were used: runx2 forward; 5'-CCAGATGGGACTGTGGTTACC-3' and reverse; 5'-ACTTGGTGCAGAGTTCAGGG-3', osterix forward; 5'-CTGGGGAAAGGAGGCACAAAGAAG-3' and reverse; 5'-GGGTTAAGGGGAGCAAAGTCAGAT-3', and GAPDH forward; 5'-ACCACAGTCCATGCCATC AC-3' and reverse; 5'-TCCACCACCCTGTTGCTGTA-3'.

Real-time RT-PCR analysis

In some experiments, the extracted total RNA was reverse-transcribed and subjected to real-time RT-PCR. For real-time RT-PCR, the PCR products were detected by the FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The following primer sequences were used: β-actin forward; 5'-CTGAACCCTAAGGCCAACCGTG-3' and reverse; 5'-GGCATACAGGGACAGCACAGCC-3', noggin forward; 5'-CTGGTGGACCTCATCGAACA-3' and reverse; 5'-CTCGTTCAGATCCTTCTCCTTAGG-3', follistatin forward; 5'-GAAAACCTACCGCAACGAATG-3' and reverse; 5'-TCCGGCTGCTCTTTGCAT-3', smad 6 forward; 5'-GGGTGTCTCTAGCATCGTTTCG-3' and reverse; 5'-CCGCGACCGCTCAACTC-3', and smad 7 forward; 5'-CAGCACTGCCAAGCATGG T-3' and reverse 5'-ACCGAAACGCTGATCCAAAG-5'. Thermal cycling and fluorescence detection were performed using a StepOne™ Real-Time PCR System (Applied Biosystems). The real-time RT-PCR efficiency (E) was calculated according to the equation provided by Rasmussen [25] (E = 10[−1/slope]) for β-actin and various target genes. The slope was determined from the graph of ng of cDNA substrate (x-axis) *versus* the cycle number at the crossing point (CP) (y-axis). The CP is the PCR cycle number that represents the CP in SYBR Green fluorescence intensity above the automatic noise-based threshold. The fold increase in copy numbers of mRNA was calculated as a relative ratio of the target gene to β-actin, following the mathematical model introduced by Pfaffl [24].

$$\text{Fold increase} = \frac{(E_{TARGET})^{\text{CP TARGET (MEAN control-MEAN subject)}}}{(E_{\beta\text{-ACTIN}})^{\text{CP }\beta\text{-ACTIN (MEAN control-MEAN subject)}}}$$

Western blot analysis

Cells were distributed in 6-well plates at a density of 8×10^5/well and incubated for 24 h. The growth medium was changed, and the cells were cultured with or without BMP-2 (100 ng/ml) and heparin (100 μg/ml). After an additional 0.5–48 h of incubation, the cells were washed with phosphate buffered saline (PBS) and lysed in lysis buffer (75 mM Tris-HCl containing 2% SDS and 10% glycerol, pH 6.8). The protein contents were measured using a DC protein assay kit. The samples were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked by immersing the membranes in 10% skim milk in PBS for 60 min at room temperature, after which the membranes were washed 4 times with PBS and incubated with the diluted primary antibody overnight at 4°C. Anti-phospho-Smad 1/5/8, anti-Smad1, anti-phospho-p38, and anti-p38 antibodies and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) were used in this experiment. After washing the membranes, ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for chemiluminescence detection with Hyperfilm-ECL (Amersham Pharmacia Biotech).

Statistical analysis

Statistical analyses for ALP activity were conducted with statistics software (JMP8.0.2, SAS Institute Inc., Cary, NC, USA). The results were expressed as the mean ± SD. One-way analysis of variance was employed to analyze the manner in which the distribution of each continuous variable differed across the groups. The Tukey-Kramer HSD (honestly significant difference) test was utilized to test differences with respect to the group means.

In real-time RT-PCR analyses, statistical significance was determined using Student's *t*-test. A p value of less than 0.05 was considered significant.

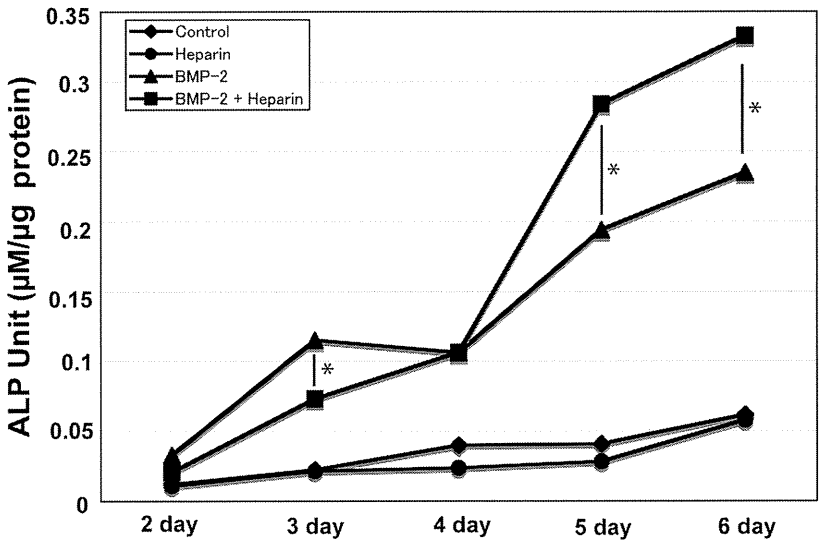

Fig. 1. Heparin has biphasic effects on ALP activity induced by BMP-2 in osteoblasts. MC3T3-E1 cells (2 10^5 cells/well) were stimulated with BMP-2 (100 ng/ml) in the presence or absence of various concentrations of heparin for 48–144 h. The specific activity of ALP was determined as described in the Materials and Methods. Values are expressed as fold increases relative to untreated controls. The data are expressed as the mean ± SD of triplicate cultures. The experiment was performed three times with similar results obtained in each experiment. * $p < 0.0001$ as measured by the Tukey-Kramer HSD test

Results

Heparin enhances osteoblast differentiation induced by BMP-2

To determine the effect of heparin on osteoblast differentiation induced by BMP-2, we assessed ALP activity, a typical marker of osteoblast differentiation. ALP activity and mineralization are well known to be dramatically enhanced when MC3T3-E1 cells are cultured with BMP-2. Heparin inhibited the ALP activity induced by BMP-2 after culturing for 72 h. However, heparin remarkably enhanced the ALP activity induced by BMP-2 after culturing for 120–144 h (Fig. 1).

Heparin sustains the BMP-2-mediated signaling activity

Next, we examined the levels of Smad 1/5/8 phosphorylation for prolonged periods of time. BMPs activate identical amino acid sequences at the C-terminal domain of R-Smads. The phosphorylation of Smad 1/5/8 was noticeable after 30 min of BMP-2 treatment, which continued for up to 6 h and then gradually decreased until 48 h. As we have previously reported [16], heparin (100 μg/ml) inhibited the levels of phosphorylation of Smad 1/5/8 induced by BMP-2 (100 ng/ml) at the time points of 30 min and 1 h. However, when the cells were incubated with both BMP-2 and heparin for

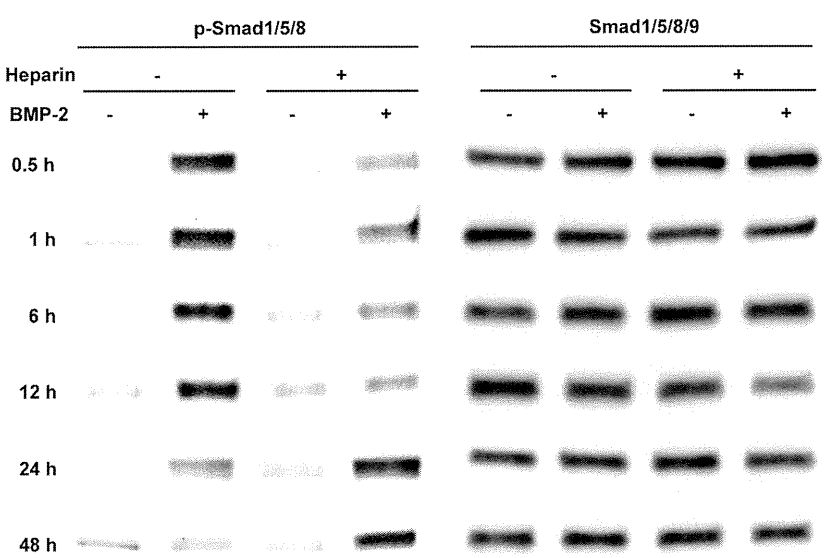

Fig. 2. Heparin has biphasic effects on BMP-2-mediated Smad-1/5/8 phosphorylation. MC3T3-E1 cells (4 10^5 cells/well) were stimulated with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 μg/ml) for the indicated time periods, then whole lysates were subjected to immunoblotting analyses

longer periods (24 and 48 h), the level of Smad 1/5/8 phosphorylation was higher than that in cells treated with BMP-2 alone (Fig. 2).

BMP receptors are well-known to determine the intensity of BMP signals *via* Smad 1 C-terminal phosphorylations, and the duration of the activated phospho-Smad signal is known to be regulated by sequential Smad linker region phosphorylation at conserved MAPK and GSK sites [8]. To elucidate the role of the p38 MAPK pathway in the regulation of BMP-2 responses, the expression of phospho-p38 MAPK was detected by Western blot analysis. However, the phosphorylation of p38 MAPK did not change when the cells were cultured with BMP-2 or heparin (data not shown).

To exclude the role of heparin in osteogenesis induced by BMP-2 for prolonged periods of time, we assessed the expression levels of genes related to osteoblast differentiation, such as runx2 and osterix, by RT-PCR. In 72-h cultures, the expression levels of runx2 and osterix mRNA were not affected by the treatment with BMP-2 (100 ng/ml). In contrast, when the cells were incubated with both BMP-2 and heparin (100 μg/ml), the mRNA expression of these genes was remarkably enhanced (Fig. 3 A, B).

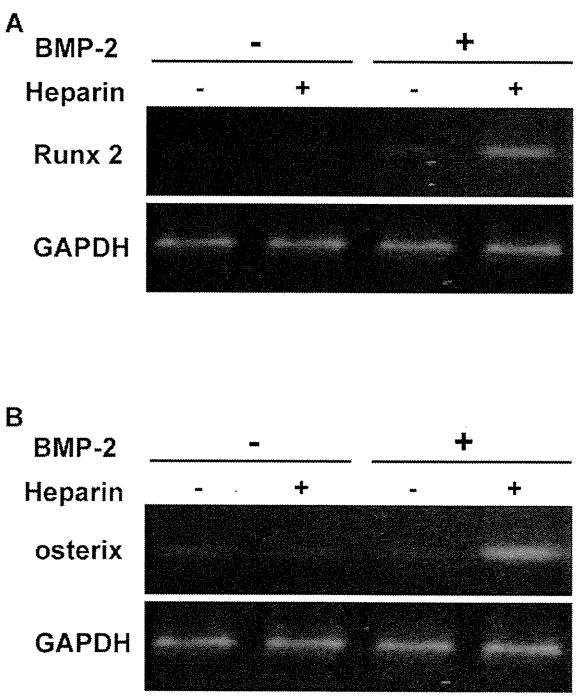

Fig. 3. Heparin enhances the gene expression of runx2 and osterix in MC3T3-E1 cells. MC3T3-E1 cells (4 10^5 cells/well) were incubated with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 μg/ml) for 72 h, then total RNA from each cell culture was reverse-transcribed with random primers. PCR amplification was performed using primers specific for (**A**) Runx2, (**B**) osterix, and GAPDH. The PCR products were resolved on 2 % agarose gels and stained with ethidium bromide

Heparin inhibits the BMP-2-induced mRNA expression of BMP-2 antagonists and inhibitory Smads

To examine the mechanisms involved in the enhancement of BMP-2-induced osteogenesis by heparin for prolonged periods of time, we assessed the expression levels of BMP-2 antagonist genes, such as noggin and follistatin, by real-time RT-PCR. In 24- and 48-h cultures, stimulation with BMP-2 (100 ng/ml) enhanced the expression levels of noggin and follistatin mRNA. In contrast, the mRNA expression of these genes was remarkably suppressed when the cells were cultured with both BMP-2 and heparin (100 μg/ml) (Fig. 4 A, B).

Finally, we assessed the expression levels of smad 6 and smad 7, which are known as inhibitory Smads, by real-time RT-PCR. In 48-h cultures, the stimulation of BMP-2 (100 ng/ml) enhanced the expression levels of smad 6 and smad 7 mRNA. However, the mRNA expression of these genes was suppressed below basal control levels when the cells were cultured with both BMP-2 and heparin (100 μg/ml) (Fig. 5 A, B).

Discussion

Long-term administration of heparin is well-known to be associated with an increased risk of developing osteoporosis [15, 36]. Heparin has also been reported to have a tendency to increase the formation of osteoclasts at lower concentrations, whereas it tends to decrease the numbers of osteoclasts in rat bone marrow cell cultures at high concentrations [7]. Recent reports have indicated that GAGs, including heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid, mediate BMP activity [14, 17, 19] and that sulfation is required for BMP activity-mediated processes [20, 21]. However, the effects of heparin (which is the most sulfated GAG) on osteogenic activity have not been fully elucidated. Takada and Zhao have indicated that heparin enhances the biological activities of

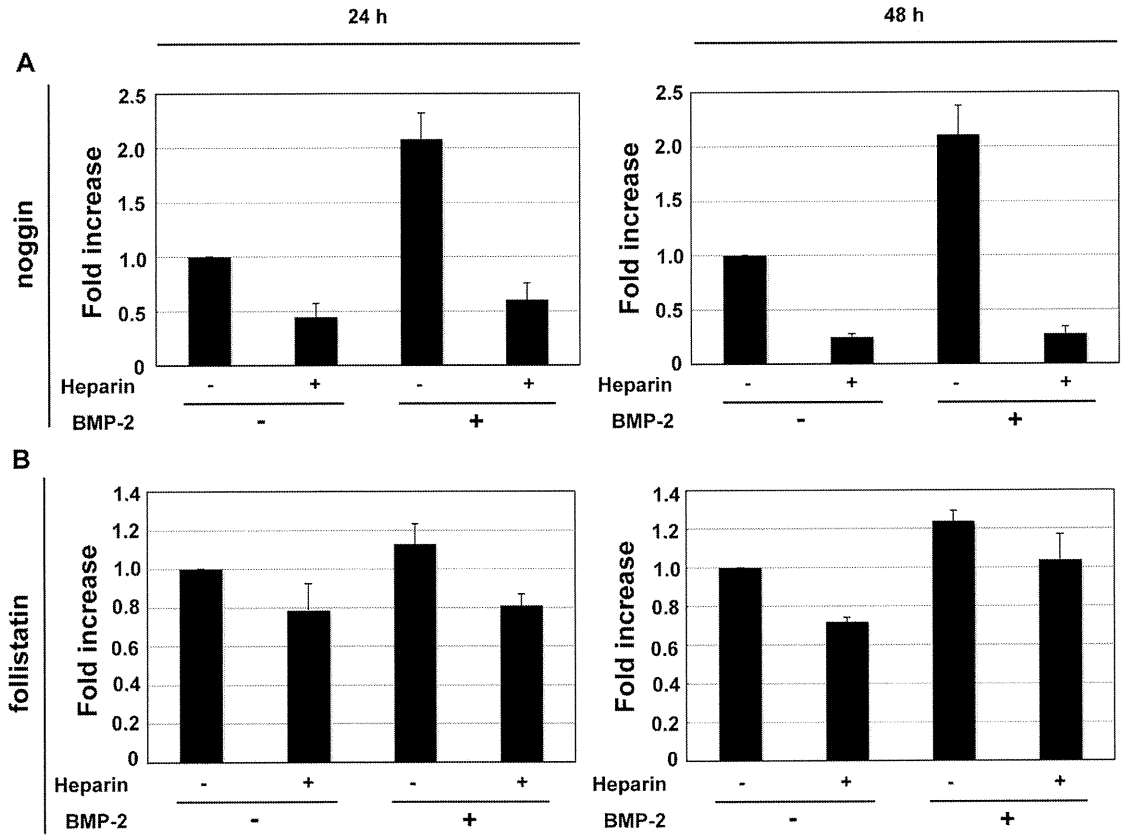

Fig. 4. Heparin suppresses the gene expression of noggin and follistatin in MC3T3-E1 cells. MC3T3-E1 cells (4 10^5 cells/well) were cultured with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 μg/ml) for 24 or 48 h. Total RNA was isolated, reverse-transcribed into cDNA and PCR-amplified using SYBR green. The PCR amplification was performed using primers specific for (**A**) noggin, (**B**) follistatin, and β-actin. The fold changes in noggin and follistatin mRNA copy number values represent the average SD of data derived from triplicate cultures. * $p < 0.05$, ** $p < 0.01$, respectively, as measured by Student's *t*-test

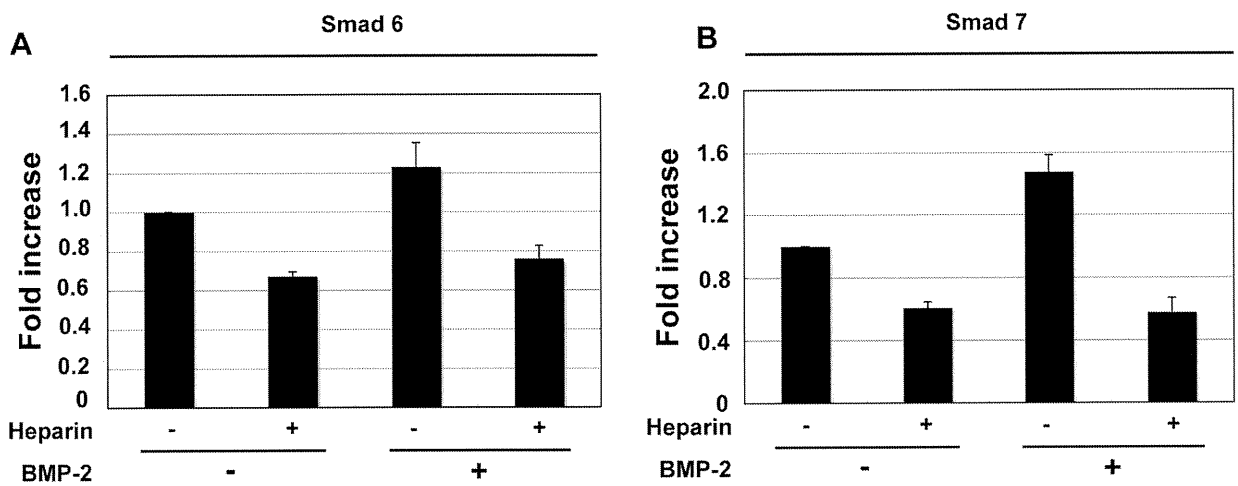

Fig. 5. Heparin supresses the gene expression of Smad 6 and Smad 7 in MC3T3-E1 cells. MC3T3-E1 cells (4 x 10^5 cells/well) were cultured with BMP-2 (100 ng/ml) in the presence or absence of heparin (100 μg/ml) for 48 h. Total RNA was isolated, reverse transcribed into cDNA and PCR-amplified using SYBR green. The PCR amplification was performed using primers specific for (**A**) Smad 6, (**B**) Smad 7, and β-actin. The fold changes in Smad 6 and Smad 7 mRNA copy number values represent the average ± SD of data derived from triplicate cultures. * $p < 0.05$, ** $p < 0.05$, respectively, as measured by Student's *t*-test

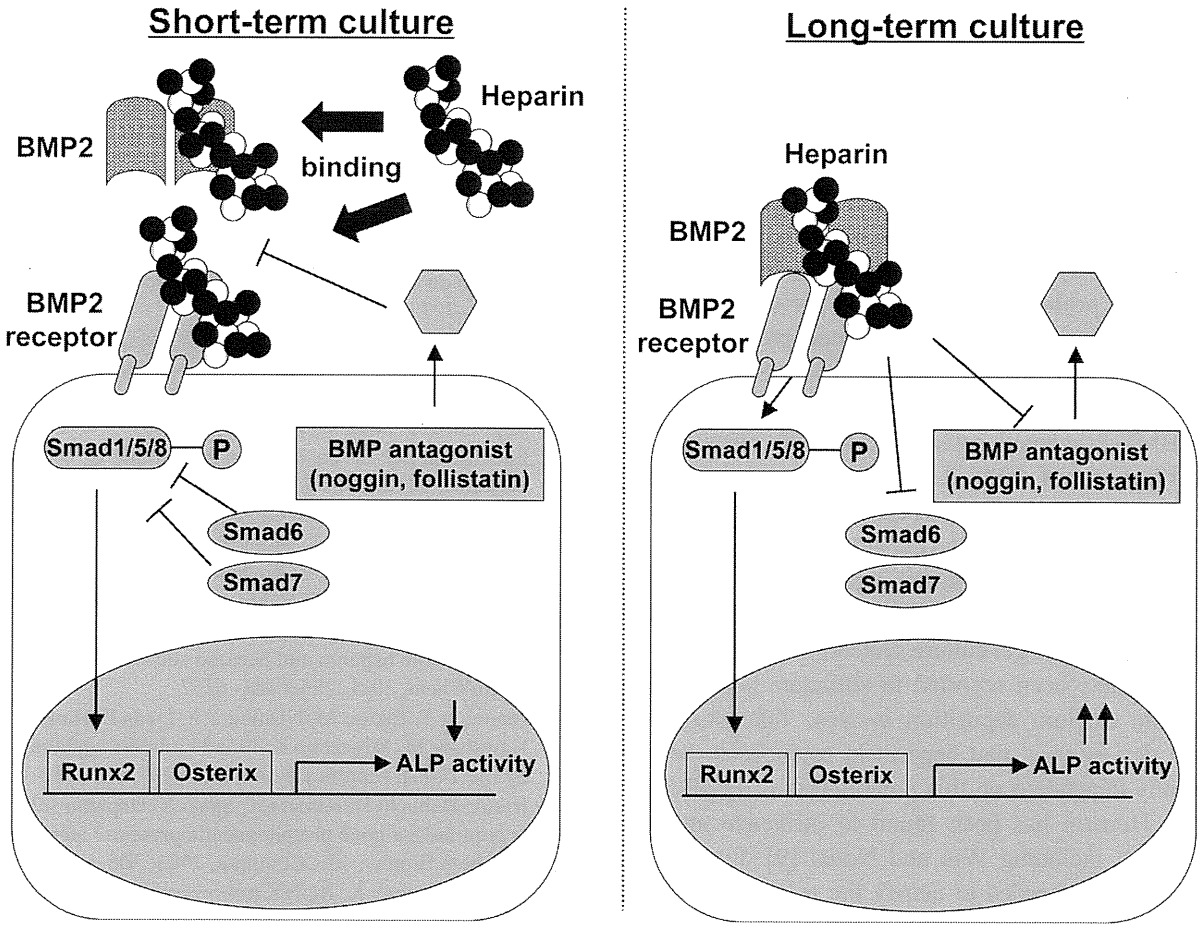

Fig. 6. Schematic of the molecular mechanism of the role of heparin in BMP-2-induced osteogenesis. In this model, short-term treatment with heparin inhibits BMP-2 osteogenic activity *via* competitive inhibition of the binding of BMP-2 to the BMP receptor. In contrast, heparin negatively regulates the negative feedback loop and enhances BMP-2- induced osteogenic activity in long- term cultures

BMP-2 by protecting BMP-2 from degradation and inhibition by BMP antagonists [32, 38]. In addition, Miyazaki has indicated that heparin alone enhances osteoblast growth, differentiation, and mineralization [20].

We have previously reported that heparin inhibits BMP-2 osteogenic bioactivities, such as ALP activity, by binding to both BMP-2 and BMPR; this binding ability of heparin also inhibits BMP-2-induced Smad 1/5/8 phosphorylation and decreases the expression levels of Runx2 and osterix genes within 12 h [16]. In the present study, we found that heparin enhanced the BMP-2 osteogenic bioactivity (Fig. 1), the phosphorylated levels of Smad 1/5/8 (Fig. 2), and the expression levels of genes related to osteoblast differentiation (Fig. 3) after a longer culture period. These data represent the first report of contrasting time-dependent effects of heparin in mediating BMP-2 activity, although previous reports have noted discrepancies between cell types, culture conditions, and heparin concentrations [6, 7, 18]. Interestingly, our results clearly indicate that time was the major factor for the discrepancy in BMP-2-mediated osteogenesis.

Previous studies have reported that bioactive BMPs remain in the extracellular space in the presence of heparin for a longer period of time and that active ligands are protected from suppression by antagonist [38]. Furthermore, our present study suggests that heparin negatively regulates the expression of BMP antagonists and inhibitory Smads that are induced by BMP signaling as part of the negative feedback loop to suppress excess signaling (Fig. 6).

We examined the mechanism by which heparin enhanced the BMP-2-mediated bioactivity for prolonged periods of time. BMP signaling is well-known to be determined by the binding of BMPs and their re-

ceptors. However, soluble BMP antagonists such as noggin and follistatin are known to directly bind to BMPs and prevent functional receptor/ligand interactions [5]. Furthermore, Smad 6 and Smad 7 have also been shown to bind to BMP receptors and inhibit BMP signaling [10, 31]. As shown in Figures 4 and 5, the mRNA expression of noggin, follistatin, Smad 6 and Smad 7 by BMP-2 was preferentially blocked by heparin in prolonged culture time periods. Although measurement of the heparin-induced protein expression of BMP-2 antagonists or inhibitory Smads is still needed, these results suggest that heparin up-regulates the BMP-2-induced osteogenic activity through the contributions of BMP-2 antagonists and inhibitory Smads. Jeon et al. have reported that heparin enhances BMP-2-induced ALP activity in rat calvarial osteoblasts using heparin-conjugated poly (L-lactide-co-glycolide) (PLGA) nanospheres (HCPNs) suspended in a fibrin gel culture system [13]. Furthermore, HCPNs have been reported to stimulate bone formation and calcium deposition *in vivo*. Taken together, these data suggest that heparin in osteoblasts may be partially dependent on this potentiation of BMP-2 activity. Heparin has been found to influence multiple pathways, including Wnt and Nodal [9]. Further experiments are needed to clarify the role of heparin in the regulation of BMP-2 both *in vivo* and *in vitro*.

In conclusion, we found that heparin inhibited BMP-2 osteogenic bioactivity in 72-h cultures and enhanced the activity in 144-h cultures. These results suggest that heparin sustains BMP-2 osteogenic activity and indicate the crucial role of heparin in bone tissue under both physiological and pathological conditions. Therefore, one might expect that the appropriate timing of heparin administration will promote bone healing mediated by BMP-2.

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RESEARCH LETTER

Streptococcus sanguinis induces foam cell formation and cell death of macrophages in association with production of reactive oxygen species

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Abstract

Streptococcus sanguinis, a normal inhabitant of the human oral cavity, is a common streptococcal species implicated in infective endocarditis. Herein, we investigated the effects of infection with *S. sanguinis* on foam cell formation and cell death of macrophages. Infection with *S. sanguinis* stimulated foam cell formation of THP-1, a human macrophage cell line. At a multiplicity of infection >100, *S. sanguinis*-induced cell death of the macrophages. Viable bacterial infection was required to trigger cell death because heat-inactivated *S. sanguinis* did not induce cell death. The production of cytokines interleukin-1β and tumor necrosis factor-α from macrophages was also stimulated during bacterial infection. Inhibition of the production of reactive oxygen species (ROS) resulted in reduced cell death, suggesting an association of ROS with cell death. Furthermore, *S. sanguinis*-induced cell death appeared to be independent of activation of inflammasomes, because cleavage of procaspase-1 was not evident in infected macrophages.

Introduction

Streptococcus sanguinis is a member of the viridans streptococci and a primary colonizer of the human oral cavity (Kolenbrander & London, 1993; Nobbs *et al.*, 2009). Viridans streptococci are known to colonize damaged heart valves and are the most frequently identified bacteria as primary etiological agents of life-threatening bacterial infective endocarditis in individuals with predisposing cardiac conditions (Douglas *et al.*, 1993; Dyson *et al.*, 1999). In this regard, epidemiological studies have shown the presence of oral streptococcal species including *S. sanguinis* in clinical specimens of heart valve and atheromatous plaque (Chiu, 1999; Nakano *et al.*, 2006; Koren *et al.*, 2011). One of the earliest events in atherogenesis is foam cell formation of blood macrophages induced by the uptake of low-density lipoprotein (LDL) (Erridge, 2008). In addition, cell death of macrophages is also considered to be associated with atherosclerosis, because dead macrophages are found in atheromatous plaque (Tabas, 2010).

Macrophages and monocytes present in the bloodstream are major contributors to host immune responses against bacterial infections. It is known that periodontal disease-related oral pathogens such as *Porphyromonas gingivalis* are involved in atherosclerosis (Hajishengallis *et al.*, 2002; Gibson *et al.*, 2005). *In vitro* studies have also shown that *P. gingivalis* elicits foam cell formation of macrophages (Qi *et al.*, 2003; Giacona *et al.*, 2004). Although *S. sanguinis* is known to induce infectious endocarditis, its possible contribution to atherosclerosis has not been studied. In the present study, we investigated

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whether *S. sanguinis* infection induces foam cell formation and cell death of human macrophages.

Materials and methods

Bacterial strains and culture conditions

Streptococcus sanguinis strain SK36 (Kilian *et al.*, 1989) was provided by Dr M. Kilian (Aarhus University, Denmark), and cultured in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, MD) supplemented with 0.2% yeast extract (Becton Dickinson). Heat-inactivated *S. sanguinis* SK36 was prepared by heating the bacterial suspension in phosphate-buffered saline (PBS; pH 7.4) at 60 °C for 30 min (Okahashi *et al.*, 2003). In some experiments, a cariogenic bacterial strain, *Streptococcus mutans* UA159, was used as a negative control.

Cell culture and foam cell formation

Human monocyte cell line THP-1 cells were purchased from RIKEN Bioresorce Center (Tsukuba, Japan) and cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen) (5% FBS RPMI1640), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}). Differentiated THP-1 macrophages were prepared by treating THP-1 cells with 100 nM phorbol myristate acetate (Sigma Aldrich, St. Louis, MO) for 2 days. For infection, differentiated THP-1 cells (5×10^4 cells in 100 μL of 5% FBS RPMI1640 without antibiotics) in 96-well culture plates (Asahi Glass, Tokyo, Japan) were infected with viable *S. sanguinis* SK36 at a multiplicity of infection (MOI) of 10, 20, or 50 for 2 h. The cells were washed with PBS to remove extracellular nonadherent bacteria, and cultured for 2 days in the presence of human LDL (100 μg mL^{-1}; Sigma Aldrich) and antibiotics. The cells were also stimulated with lipopolysaccharide (LPS) of *Escherichia coli* O127 (Sigma Aldrich) or heat-inactivated *S. sanguinis* SK36 whole cells for 2 days. The macrophages were fixed with 10% formaldehyde, and stained for 15 min with 1% oil-red O (Sigma Aldrich) in 60% isopropanol (Qi *et al.*, 2003; Giacona *et al.*, 2004). Approximately 100 cells per well were examined using a microscope (×200) (Nikon DIAPHOT TMD 300; Nikon, Tokyo, Japan).

Adhesion and internalization

Differentiated THP-1 macrophages were infected with viable *S. sanguinis* SK36 (MOI; 50, 100 or 200) or *S. mutans* UA159 in the absence of antibiotics. After 2 h of incubation, the cells were washed three times with PBS, and were disrupted by vortexing with sterile water. Serial dilutions of the cell lysates were plated onto BHI agar plates to determine the number of adherent bacteria (CFU). For the internalization assay, the extracellular adherent bacteria were killed by incubating with gentamicin (100 μg mL^{-1}) and penicillin G (100 U mL^{-1}) for 1 h. The cells were then lysed with sterile water and CFU of intracellular bacteria were counted on BHI agar plates (Okahashi *et al.*, 2003).

Cell death of infected macrophages

Differentiated THP-1 macrophages (2×10^5 cells in 5% FBS RPMI1640) were infected with viable *S. sanguinis* SK36 (MOI 50, 100 or 200) or heat-inactivated *S. sanguinis* (MOI 500 or 1000) in the absence of antibiotics for 2 h. The cells were washed with PBS and cultured for 18 h in fresh medium containing antibiotics. The cells were then stained with 0.2% trypan blue (Sigma Aldrich) in PBS. After incubation at room temperature for 5 min, the numbers of viable and dead cells were counted using a microscope (Nikon TMS-F; Nikon).

Confocal microscopy

Differentiated THP-1 cells were cultured on gelatin-coated coverslips in 24-well culture plates. The macrophages were exposed to *S. sanguinis* SK36 at an MOI of 200 for 2 h, washed with PBS to remove extracellular bacteria, and cultured for a further 6 h. Prolonged incubation resulted in detachment of the dead macrophages from the coverslips. Uninfected cells were used as a negative control. The cells were first stained with propidium iodide (PI) (Sigma Aldrich), washed with PBS, treated with 0.1% Triton X100 in PBS for 10 min, and then stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Aldrich). The stained cells were analyzed using an LSM 510 confocal laser microscope (Carl Zeiss, Oberkochen, Germany). PI stained the nuclear DNA of dead THP-1 cells, whereas DAPI stained that in all cells.

Cytokine assay

Differentiated THP-1 macrophages were infected with viable *S. sanguinis* SK36 (MOI 50, 100 or 200) or heat-inactivated *S. sanguinis* (MOI 500 or 1000) in the absence of antibiotics for 2 h. The cells were washed with PBS to remove extracellular bacteria, and cultured in fresh medium containing antibiotics for a further 18 h. As a stimulant, *E. coli* LPS (1 μg mL^{-1}) was also utilized. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the culture supernatants were measured using enzyme-linked immunosorbent assay kits (ELISA; Thermo Fisher