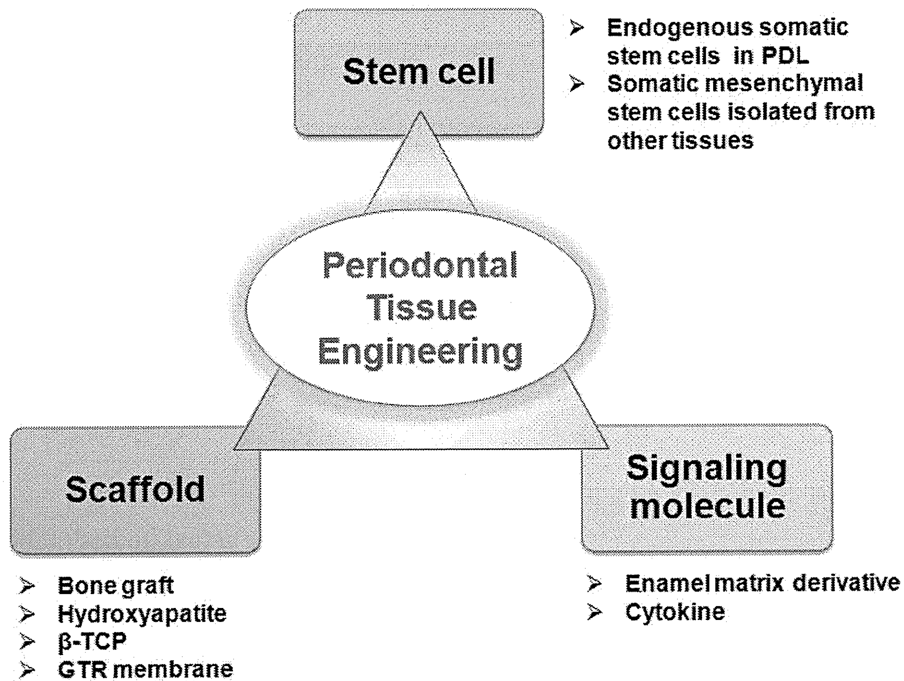

Figure 1. Triad of periodontal tissue engineering. The concept of tissue engineering consists of stem cells, a signaling molecule and a scaffold. In the case of periodontal tissue engineering, the above-indicated stem cells, signaling molecules and scaffold materials have been examined pre-clinically, with some having already been introduced into clinics.

1.	PDGF-BB (platelet-derived growth factor) plus IGF-I (insulin-like growth factor-I)
2.	BMP-2 (bone morphogenetic protein-2)
3.	TGF-β (transforming growth factor-β)
4.	OP-1 (BMP-7) (osteogenic protein-1)
5.	BDNF (brain-derived neurotrophic factor)
6.	FGF-2 (bFGF) (basic fibroblast growth factor
7.	PDGF-BB (platelet-derived growth factor) plus β-TCP (GEM21S™) (β-tricalcium phosphate)
8.	GDF-5 (growth and differentiation factor-5)

Table 1. Periodontal regeneration by recombinant cytokines

applying topical FGF-2 to induce periodontal tissue regeneration, a series of animal studies using beagle dogs and non-human primates was performed (Murakami *et al* 2003c, Takayama *et al* 2001c). The mandibular molars of beagle dogs, and the first and second molars of non-human primates, were utilized for experimentation. After elevation of mucoperiosteal flaps, class II furcation defects were surgically created and the exposed cementum removed by curettage, before vinyl polysiloxane impression material was placed in the defects to induce inflammation. Four weeks after the first surgery, a flap was raised to expose the inflamed furcation, granulation tissues were removed and the root surfaces curetted. A small round bur was used to make a horizontal groove on each root in order to indicate the base of the defect. Furcation defects were filled with a gelatinous carrier without or with FGF-2 and the wound was surgically closed. Periodontal tissue regeneration at the test sites of beagle dogs and non-human primates was examined at 6 and 8 weeks respectively, after FGF-2 application to the defects.

As shown in Tables 2 and 3, topical application of FGF-2 significantly stimulated periodontal regeneration in both the beagle and the non-human primate models when compared to control sites (Figure 2). Histological observation revealed new cementum with Sharpey's fibers, new functionally-oriented periodontal ligament fibers and new alveolar bone (Murakami *et al* 2003a, Takayama *et al* 2001). Interestingly, enhancement of angiogenesis and regeneration of peripheral nerve fibers at the FGF-2-treated sites were also observed one week after FGF-2 application (Murakami 2011a).

More importantly, no epithelial

	Control site (n=6)	**0.1% FGF-2-applied site (n=6)**
NBF (%)	35.4 ± 8.9	83.6 ± 14.3*
NTBF (%)	16.6 ± 6.2	44.1 ± 9.5*
NCF (%)	37.2 ± 15.1	97.0 ± 7.5*

*: $p<0.01$, Control site - gelatinous carrier alone was applied.

Table 2. Efficacy of FGF-2 for periodontal tissue regeneration in animal models - Furcation class II model in beagle dogs (6-week follow up) (modified from Murakami *et al* 2003)

	Control site (n=6)	**0.4% FGF-2-applied site (n=6)**
NBF (%)	54.3 ± 8.0	71.3 ± 13.5*
NTBF (%)	31.6 ± 3.5	48.7 ± 8.9**
NCF (%)	38.8 ± 8.6	72.2 ± 14.4**

*: $p<0.05$, **: $p<0.01$, Control site - gelatinous carrier alone was applied.

Table 3. Efficacy of FGF-2 for periodontal tissue regeneration in animal models - Furcation class II model in non-human primates (8-week follow up) (modified from Takayama *et al* 2001)

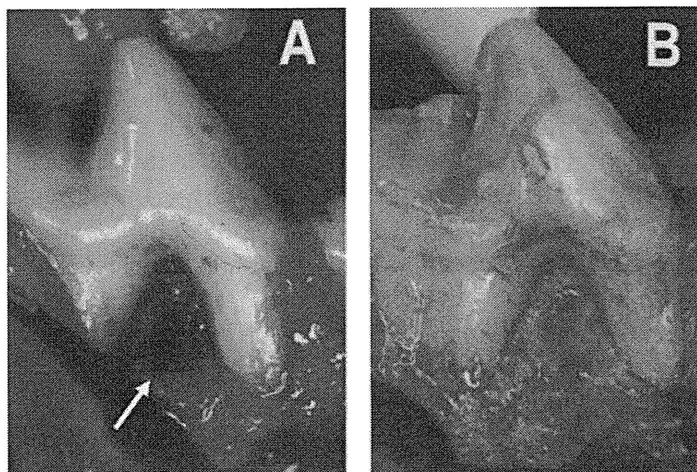

Figure 2. Periodontal tissue regeneration by FGF-2 (furcation class II beagle model).
FGF-2 (0.1%) plus gelatinous carrier was topically applied to surgically-created class II furcation defects in the mandibular molars of beagle dogs. Representative images at (A) baseline and (B) 6 weeks after FGF-2 application are shown. Arrow indicates furcation. (from Murakami *et al* 2003)

downgrowth, ankylosis or root resorption was observed at the FGF-2 sites in any of the *in vivo* experiments, nor was any severe gingival inflammation or swelling observed at any of the sites examined throughout the experimental periods.

In vitro analyses of effects of FGF-2

It has already been demonstrated that FGF-2 promotes proliferation of fibroblasts and osteoblasts, and enhances angiogenesis. These activities are crucial in the process of periodontal tissue regeneration. However, periodontal ligament (PDL) cells also play an important role during periodontal tissue regeneration (Seo *et al* 2004, Lekic *et al* 2001, Murakami *et al* 2003b, Shimono *et al* 2003). To reveal the molecular and cellular mechanisms by which FGF-2 enhances periodontal tissue regeneration, a series of *in vitro* experiments using PDL cells were carried out.

RT-PCR experiments demonstrated that PDL cells express FGF receptor (FGFR) 1 and FGFR2 mRNA (Takayama *et al* 2002), and diminished immature PDL cells begin to

differentiate into osteoblasts and cementoblasts.

FGF-2 also stimulated significant migration of PDL cells, even when their proliferation was completely inhibited by mitomycin-C. Furthermore, it was shown that FGF-2 stimulates the biosynthesis of hyaluronan (HA) and the cell surface expression of CD44, and that the interaction between these molecules plays a crucial role in PDL cell migration (Shimabukuro *et al* 2010).

This series of *in vitro* studies has facilitated the development of a hypothesis on the mode of action of FGF-2. Thus, during the early stages of periodontal tissue regeneration, FGF-2 stimulates proliferation of PDL cells while suppressing their differentiation (Figure 3). Then, during the subsequent healing process, when FGF-2 is no longer present at the administration site, PDL cells begin to differentiate into hard-tissue-forming cells such as osteoblasts and cementoblasts resulting in marked periodontal tissue regeneration at sites of FGF-2 application. In addition, FGF-2 induces the angiogenesis that is indispensable in the regeneration of tissue, and regulates the production of osteopontin, heparan sulfate and HA from PDL cells (Takayama *et al* 1997, Shimabukuro *et al* 2005, Shimabukuro *et al* 2008, Terashima *et al* 2008). Notably, FGF-2 specifically promotes the production of high molecular weight HA, which plays an important role in cell migration and the early stages of wound healing (Shimabukuro *et al* 2005). Based on the results described above, we concluded that FGF-2 contributes to the overall regeneration

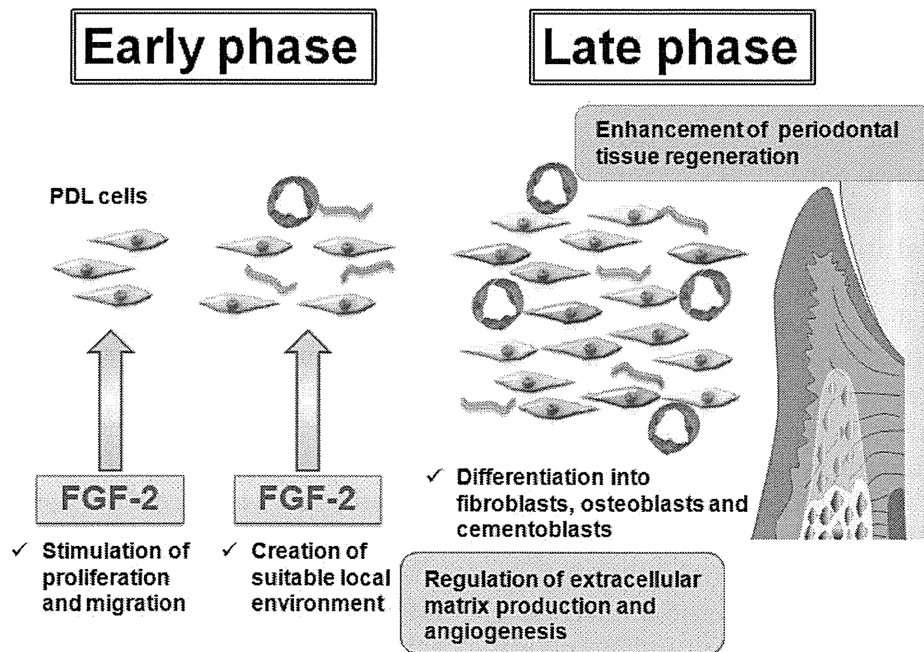

Figure 3. Possible mode of action of FGF-2 in induction of periodontal regeneration.
During the early stages of periodontal tissue regeneration, FGF-2 stimulates the proliferation and migration of PDL cells while maintaining their multipotent nature, and in later stages induces their differentiation into hard-tissue-forming cells such as osteoblasts and cementoblasts. Furthermore, FGF-2 induces angiogenesis and increases the production of osteopontin, HS and macromolecular HA from PDL cells, creating a local environment suitable for the regeneration of periodontal tissue.

of periodontal tissue by creating a local environment that facilitates the function of this mechanism (Murakami 2011b).

Clinical trial of FGF-2 for periodontal tissue regeneration

Phase IIA clinical trial

Given the promise shown by FGF2 as a periodontal regeneration agent, we performed a Phase II clinical trial. Using data from animal trials, we estimated that an effective FGF-2 concentration for periodontal tissue regeneration is 0.03 to 0.3%. This concentration range was therefore applied in the Phase IIA trial.

We prepared gel-like investigational drugs using 3% hydroxypropylcellulose (HPC) as a vehicle. We then designed a double-blinded clinical trial with approximately 80 periodontitis patients from 13 dental facilities in Japan. Patients displaying a two- or three-walled vertical bone defect $\geq$3 mm from the top of the alveolar bone were registered for this clinical trial and randomly divided into four groups: Group P (Placebo), Group L (0.03% FGF-2), Group M (0.1% FGF-2) and Group H (0.3% FGF-2). Patients underwent flap surgery during which we administered 200 μl of the appropriate investigational drug to periodontal tissue defects. For efficacy analysis, standardized radiographs of the region of investigation were taken before and 36 weeks after administration of the investigational drug. The rate of increase in alveolar bone height was independently measured by five specialist dental radiologists who were blinded to the treatment each patient had received. The median of five measurements taken from the same image was then selected for efficacy analysis.

We observed that the mean alveolar bone height in Group H (0.3% FGF-2) gradually increased for 36 weeks after application (Figure 4). After 36 weeks, a significant increase (p=0.021) in alveolar bone height was seen on standardized radiographs between Group P (23.92%) and Group H (58.62%) (Figure 4) (Kitamura *et al* 2008). No serious adverse effects were seen during the course of this clinical trial. The data obtained from this clinical trial suggest that topical application of FGF-2 is efficacious in regenerating periodontal tissue in patients with two- or three-walled intrabony defects.

Phase II B clinical trial

Having obtained positive results from the Phase IIA trial, we progressed to a Phase IIB trial (Kitamura *et al* 2011). In this large clinical trial, approximately 260 periodontitis patients from 25 dental facilities in Japan were registered, and were randomly divided into four groups comprising a placebo group and three FGF-2 groups (0.2, 0.3 and 0.4%). Results, in terms of efficacy and safety, were similar to the Phase IIA trial (Kitamura *et al* 2011, Murakami *et al* 2011a).

However, in both the Phase IIA and IIB trials, no significant differences in the regain of clinical attachment loss (CAL) between Group P and the FGF-2 groups were found. This is in agreement with observations reported in a clinical trial showing the efficacy of PDGF-BB plus β-TCP for periodontal regeneration (Nevins *et al* 2005). We speculate that differences may exist between Group P and the three FGF-2 groups in the histological ratio of fibrous and epithelial attachments achieving CAL acquisition.

Future Outlook of FGF-2 therapy

"Tissue engineering" is a fundamental concept in tissue regeneration. As mentioned above, we observed that topical application of FGF-2 significantly induces periodontal tissue regeneration, including fibrous attachment

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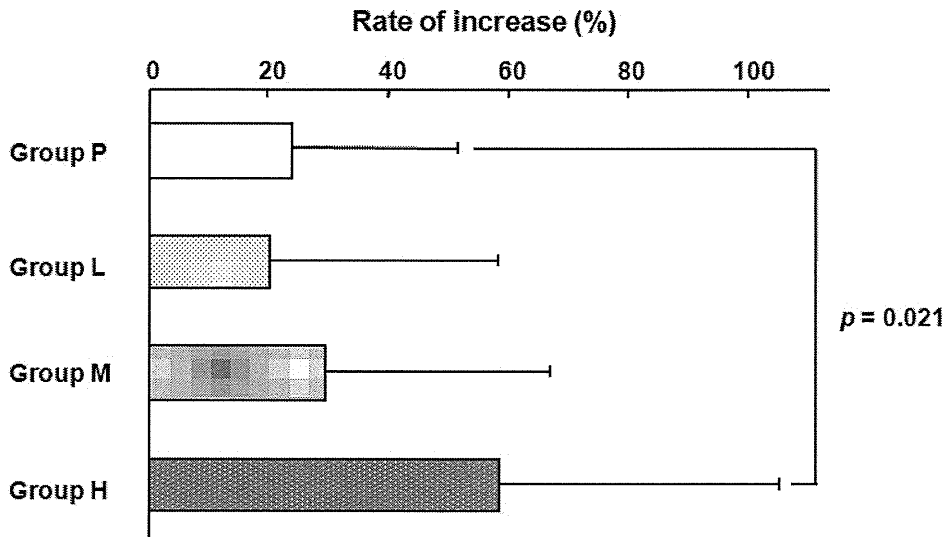

Figure 4. Rates of increase in alveolar bone height in cases of 2- and 3-walled intrabony defects.
We compared rates of increase in alveolar bone height at 36 weeks after FGF-2 administration among Group P (Placebo; n=19), Group L (0.03% FGF-2; n=19), Group M (0.1% FGF-2; n=19) and Group H (0.3% FGF-2; n=17). Graph shows mean rates of increase in alveolar bone height (%) ± standard deviation. While no significant difference was observed between Groups L, M and P, Group H showed significantly ($p = 0.021$) increased alveolar bone height in the bone defect region compared to Group P. (Modified from Kitamura *et al* 2008)

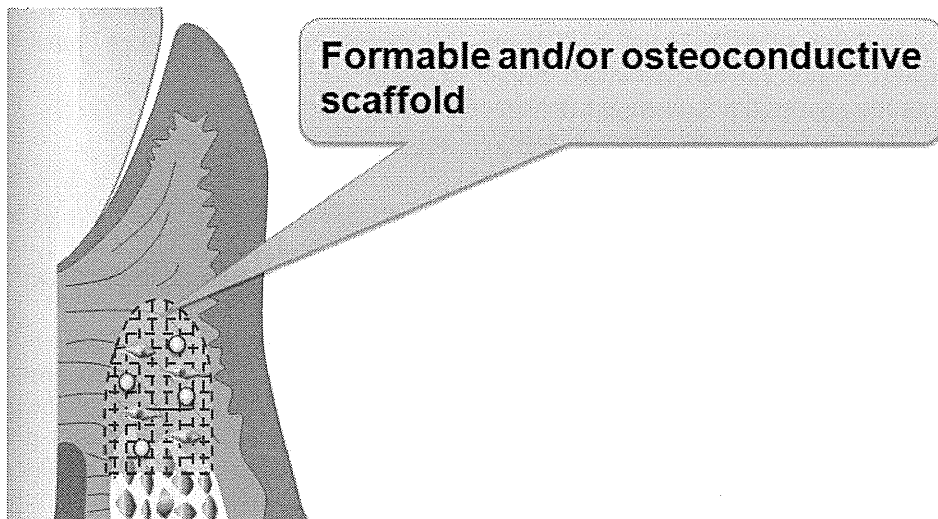

Figure 5. Ideal FGF-2 carrier for periodontal tissue regeneration.
An FGF-2 carrier that could provide a formable and osteoconductive scaffold for undifferentiated cell types within periodontal ligament would dramatically increase the indications of an FGF-2-based drug.

and neogenesis of alveolar bone and cementum in animal models. It is also noteworthy that no gingival epithelial downgrowth was observed at sites to which FGF-2 was applied. In the clinical trials of 0.3% FGF-2, we observed significant differences in the rate of increase in alveolar bone height between the placebo group and the FGF-2 group (Kitamura *et al* 2008, Kitamura *et al* 2011). This suggests that FGF-2 is efficacious for periodontal regeneration of intraosseous bone defects such as 2- or 3-walled bone defects and probably furcation involvements. However, to treat severe bony defects such as 1-wall or horizontal bone defects with FGF-2, the FGF-2 carrier may require the function of a "scaffold" to reinforce/direct its actions. HPC, which was used in our clinical trials as a carrier, does not function as a scaffold. Development of an FGF-2 carrier that provides a formable and osteoconductive scaffold for undifferentiated cell types would dramatically increase the indications of FGF-2 drugs beyond dental applications and into the craniofacial field (Figure 5). We recently examined the combined effects of FGF-2 and β-TCP on periodontal regeneration in 1-wall bony defects in beagle models and found that the combination induced significant periodontal tissue regeneration, compared with β-TCP alone (Anzai *et al* 2010). This suggests that the combination of scaffold material(s) and bioactive molecule(s) such as FGF-2 could be useful for the treatment of severe cases.

The efficacy of "cytokine therapy" in periodontal tissue regeneration was first reported in the 1990s. Since then, various recombinant cytokines have been investigated for their efficacy (and safety) in stimulating periodontal tissue regeneration, however few have been approved for use in the dental field. Therefore, we need to evaluate carefully the usefulness and safety of cytokine therapy in stimulating periodontal tissue regeneration. We hope that our work, together with future investigations, will provide a framework within which to understand "cytokine therapy" and its application to periodontal regeneration and oral reconstruction. Furthermore, "stem cell therapy" may also assist in improving periodontal regenerative therapy. It has already been reported that transplantation of bone marrow-derived cells or adipose-tissue derived stem cells can enhance periodontal regeneration (Murakami 2011b). The combined effects of "cytokine therapy" and "stem cell therapy" still require investigation.

Acknowledgments

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エンドトキシン研究 14 Brief Report

表題：バルプロ酸は，HMGB1の能動放出を誘導して，エンドトキシンショックに対する感受性を高める。

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はじめに

敗血症は，細菌感染を基盤とする全身性炎症反応症候群であり，ショックや多臓器不全に移行する重篤な病態である。敗血症を契機に，米国では毎年約 22 万人，本邦では毎年推定 5 万人以上が死亡しており，抗菌剤や抗炎症剤が発達した現在においても，集中治療領域において重大な死因となっている。敗血症の病態は炎症性サイトカインが過剰に産生され，全身に及んだ制御不能な炎症反応である。

1999 年，Wang らは，High Mobility Group Box 1 (HMGB1)が致死的エンドトキシン血症や敗血症の重要なメディエーターであると報告した。Wang らは，エンドトキシンショックや敗血症モデルマウスを用いて, 血中の HMGB1 濃度が発症後 16~32 時間後に上昇することを報告した。またマウスへの組換え HMGB1 の投与は，発熱や組織破壊を誘導し敗血症の病態を誘発することや, 抗 HMGB1 抗体はエンドトキシンショックマウスや敗血症マウスの生存率を改善した。以上より，HMGB1 が敗血症において重要な役割を演じている事を明らかとした。

1. HMGB1

HMGB1 は，1976 年に仔牛の胸腺より同定された 215 アミノ酸残基から構成されるタンパク質である。その C 末端側は負電荷の acidic tail が存在し，N 末端側は約 70 アミノ酸残基の ”box A，box B”を構成しており DNA 結合部位がある。また核移行シグナル （Nuclear localization signal : NLS）を有しており，NLS がアセチル化され核と細胞質間を移行していると考えられている。

HMGB1 は，多彩な細胞の核内に普遍的に存在する非ヒストンタンパク質である。

核内ではタンパク質複合体を形成しクロマチン構造の安定性を保持し，様々な遺伝子の転写制御，及びDNA損傷の修復に関わっている。

一方，通常核内に存在するHMGB1が2通りの機構により細胞外へと放出されることが明らかとなってきた。それは，壊死した細胞から放出される受動放出（passive release)と，活性化したマクロファージや単球から放出される能動放出（active secretion)である。細胞外環境に放出されたHMGB1は，Receptor for advanced glycation end products (RAGE)やToll-like receptor (TLR) 2/4にシグナルを伝えると考えられている。更に興味深いことに，HMGB1はLipopolysaccharide (LPS)と親和性を示し，RAGEを介してLPSによるサイトカイン産生を著しく亢進することが報告された。また，CpG DNAなど免疫原性を持つ核酸が，受容体であるTLR 3/9へシグナルを伝える際に，HMGB1は必須なタンパク質であることも明らかとなった。すなわち，HMGB1は細胞内外でTLRのリガンドと共役し，TLRシグナル伝達に重要な役割を担っている事が示唆される。

2. GABAergic

Valproic acid （VPA）は，抗てんかん薬，あるいは躁病等の気分障害の治療薬として広く用いられている薬物である。現在，VPAの作用機序は，抑制性シナプスにおいてGABA分解酵素を阻害しGABAの作用を増強することにより抗痙攣作用を発揮すると考えられている。2001年，Phielらは VPAが*in vitro*でHistone deacetylase (HDAC) 1，2，3，4，8を選択的に阻害することを報告した。またVPAにより，Trichostatin A (TSA)などの既知のHDAC阻害薬と同様にヒストンのアセチル化が亢

進することを明らかにした。更に，VPA は HDAC の制御を介し，GABA 受容体の発現を制御することも報告された。すなわち，VPA の作用機構に HDAC の活性が深く関わっていることが示唆される。

近年，GABA 受容体は神経細胞のみならず免疫系細胞や幹細胞にも発現していることが明らかとなってきた。幹細胞においては，GABA-GABA 受容体シグナルは，細胞増殖能力を制御していることが報告された。また，マクロファージに発現している GABA 受容体は，免疫応答を制御し，GABA-GABA 受容体シグナルは，IL-6 及び IL-12 の発現を制御することも報告された。以上のことから，VPA は，脳神経系のみならず全身性に影響を与えると考えられる。

3. Valproic acid induced HMGB1 acrive release.

HDAC は，ヒストンのみならず核内タンパク質のアセチル化修飾を制御していることが知られている。また HMGB1 はアセチル化修飾され核から細胞質へ移行する。しかし，HDAC 阻害剤と HMGB1 の動態の詳細な報告は少ない。そこで，われわれは HDAC 阻害剤 VPA による RAW 細胞における HMGB1 の動態を調べた。その結果，VPA により RAW 細胞から細胞障害を伴わずに HMGB1 が細胞外に Active secretion されることが明らかとなった。また 2010 年，John らは，肝細胞において HDAC 阻害剤 TSA は，HDAC1，4 の抑制を介し HMGB1 の放出を誘導することを報告した。以上のことから，HMGB1 の放出には，HDAC が関与している可能性が示唆される。

また HMGB1 の放出機序として，MAPK 経路を介していることが数多く報告されている。2007 年，Daolin らは，Hydrogen peroxide は，JNK 及び ERK を介し，RAW

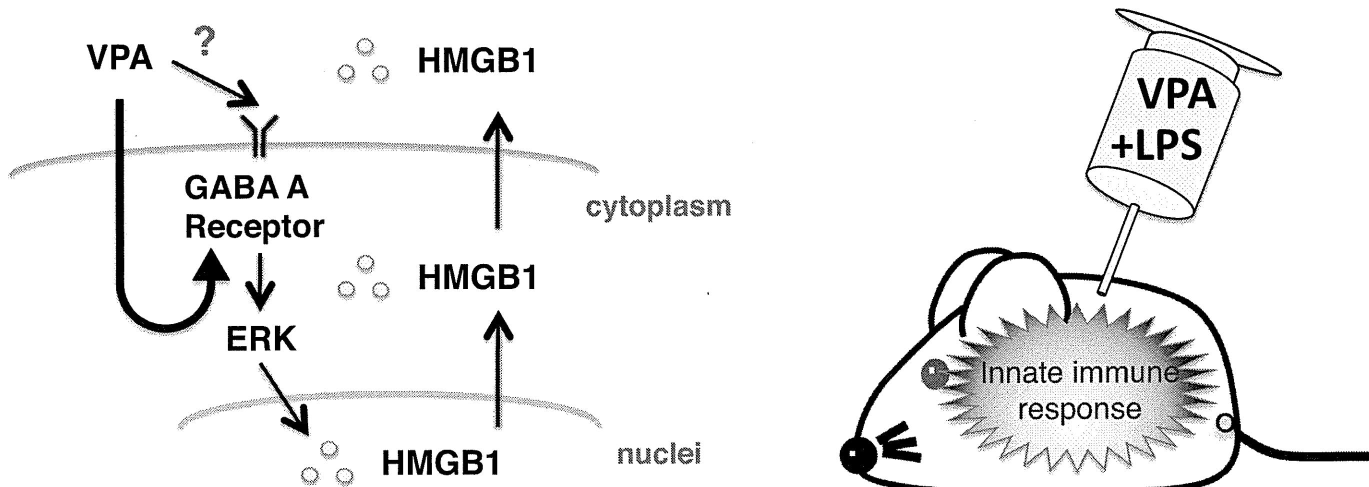
細胞および単球から HMGB1 を放出すると報告した。また 2008 年，川原らは，C-reactive protein は，RAW 細胞から p-38 を介し HMGB1 を放出すると報告した。更に，VPA は，一部の細胞種の MAPK 経路を活性化するとの報告がある。そこでわれわれは，VPA 誘導性の HMGB1 の放出機序を検討した。その結果，VPA により RAW 細胞の MAPK 経路のうち ERK の活性化のみが誘導させていることが明らかとなった。更に ERK の阻害剤により，濃度依存的に VPA 誘導性の HMGB1 の放出が阻害された。VPA は，マクロファージの GABA 受容体の発現を制御するとの報告がある。われわれは，VPA による GABA 受容体の発現を RT-PCR により検討したところ，$GABA_A$ 受容体の$\alpha1$，$\alpha3$ サブユニットの発現が誘導されていることが明らかとなった。そこで $GABA_A$ 受容体の特異的阻害剤 Pictoroxin (PTX)を用いたところ，PTX により VPA による ERK の活性化が阻害された。加えて PTX により VPA 誘導性の HMGB1 の放出が抑制された。すなわち，VPA は $GABA_A$ 受容体の発現を誘導し，ERK の活性化を介し HMGB1 の放出を誘導することが明らかとなった。

4. Valproic acid increased susceptibility to endotoxin shock.

HDAC 阻害剤がエンドトキシンショックマウスや，敗血症モデルマウスに与える影響は様々な報告がある。2009 年に，Li らは，TSA は敗血症モデルマウスの肝障害及びサイトカイン産生を抑制し，マウスの延命効果につながると報告した。また 2010 年に，Shang らは，VPA が敗血症モデルラットの多臓器障害を軽減するとの報告をした。2011 年には，Thierry らは，VPA は，サイトカイン産生を減少させ，細菌性ショックマウスの生存率を低下させる一方，腹膜炎モデルマウスの生存率を回復させ

ると報告した。しかし，HDAC 阻害剤による HMGB1 とエンドトキシンショックマウスとの関連性を検討した報告は無い。

そこで，われわれは，まず VPA によるエンドトキシンショックマウスへの影響を検討した。VPA+LPS 投与群の生存率は，LPS 単独投与群に比べ，有意に減少した。エンドトキシンショックは，著しく過剰な炎症反応として特徴づけられるので，血清中のサイトカイン濃度を測定した。エンドショック誘導後，24 時間後の血清中の HMGB1 濃度は，VPA+LPS 投与群で，LPS 単独投与群に比べ増加していた。近年，HMGB1 はエンドショックの主要因子として知られている。そこで，VPA+LPS 投与群に抗 HMGB1 中和抗体を投与し検討した。その結果，コントロール中和抗体投与群に比べ，抗 HMGB1 中和抗体投与群で有意な生存率の回復を認めた。



Proposed scheme for VPA induction of HMGB-1 release：VPA induces HMGB1 release through GABAergic signal transmission

5．まとめ

VPA は，HDAC 阻害作用が報告された後，抗癌作用や抗炎症作用など多岐にわたる作用が明らかになってきて，現在非常に注目されている。本稿で，われわれは VPA によるマクロファージ様細胞からの HMGB1 の放出及び，VPA による HMGB1 を介したエンドトキシンショックマウスの生存率の低下を報告した。HMGB1 は，TLR のリガンドと共存する際，過剰なシグナル伝達の為に炎症反応を亢進すると報告されている一方，HMGB1 は細胞増殖や細胞誘導，組織修復にも働くとの報告がある。つまりコントロールされた HMGB1 の存在は，生体に対し優位に機能すると考えられる。すなわち VPA による制御下の HMGB1 の存在は，生体に有利に働くことが十分期待出来ると考えられる。今後は，更なる HMGB1 の機能の解明を通じ，炎症のコントロールや，敗血症の病態解明や治療方法の進歩を期待してやまない。

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Morroniside Derivative Regulates E-Selectin Expression in Human Endothelial Cells

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Abstract. E-selectin is particularly of interest in the case of inflammatory diseases owing to its expression in the activated endothelium. E-selectin mediates cell tethering and rolling interactions through the recognition of sialofucosylated lewis carbohydrates expressed on circulating leukocytes. This phenomenon serves as an important trigger in inflammatory response. We prepared three morroniside derivatives and harpagoside as a positive control and then examined the effects of these compounds on E-selectin expression in human endothelial cell cultures. We found that 7-*O*-cinnamoylmorroniside significantly suppressed the expression of E-selectin induced with TNF-α (IC_{50}=49.3 μM). Furthermore, it was more active than another cinnamic-acid-conjugated iridoid glycoside (harpagoside;

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IC_{50}=88.2 μM), 7-*O*-methylmorroniside, and morroniside itself. These results suggest that 7-*O*-cinnamoylmorroniside is a potent inhibitor of TNF-α-induced E-selectin expression and that it may be useful as an anti-inflammatory agent.

Key words. Cell-ELISA, E-selectin, Flavonoid

1 Introduction

Flavonoids, such as flavones, flavonols, flavanones, flavanonols, flavans, flavanols, leucoanthocyanidins, anthocyanidins, aurones, chalcones, and isofavones, are polyphenolic compounds that occur ubiquitously in plants and have a variety of biological effects both in vitro and in vivo. They have been found to have anti-inflammatory activity in both proliferative and exudative phases of inflammation [1].

One of the initial events in inflammation is activation of endothelial cells, which then express cell surface adhesion molecules such as the endothelial leukocyte adhesion molecule (E-selectin) [2, 3]. Inflammatory cytokines such as tumor necrosis factor α (TNF-α) activate endothelial cells to express adhesion molecules and promote synthesis and release of a variety of inflammatory cytokines and chemokines to thereby support recruitment of activated leukocytes to an inflammatory lesion [4].

In this study, we prepared several morronside derivatives and examined their anti-inflammatory effect by examining E-selectin expression on human endothelial cells in vitro.

2 Materials and Methods

Human umbilical vain endothelial cells (HUVECs) (4×10^4 cells/well) were incubated with 10 ng/mL of TNF-α in the presence and in the absence of test specimens for 2 h. The expression level of E-selectin in HUVECs was measured by an ELISA.

3 Results and Discussion

7-*O*-Cinnamoylmorroniside exhibited excellent anti-inflammatory activity (IC_{50}=49.3 μM) by inhibiting the expression of E-selectin, and it was more active than another cinnamic-acid-conjugated iridoid glycoside (harpagoside; IC_{50}=88.2 μM), 7-*O*-methymorroniside, and morroniside itself. As a result, 7-*O*-cinnamoylmorroniside was observed to be a potent inhibitor of TNF-α-induced E-selectin expression.

In this study, we showed that 7-*O*-cinnamoylmorroniside markedly attenuated E-selectin adhesion to TNF-α-activated endothelial cells and that it may inhibit monocyte adhesion on the activated endothelium, thus conferring protection against atherogenic lesion formation. Thus, the compound may hamper initial atherosclerotic events involving endothelial CAM induction.

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