書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
八重垣健	ラクトフェリン+ラクトパーオキシダーゼ配合錠菓の口臭抑制効果と唾液中細菌に対する影響	日本ラクトフェリン学会編	ラクトフェリン 2011	日本医学館	東京	2011	59-63
Yaegaki K	Induction of apo-ptosis in humankeratinocyte stem cells: the hydrogen sulfide	Hayat MA	Stem Cells and Cancer Stem Cells: Therapic Application in Disease and Injury	Springer	New York	2011	371-376
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Tatsuji Nishihara	Apoptosis and survivability of human dental pulp cells under exposure to Bis-GMA	J Appl Oral Sci.	19(3)	218-22	2011

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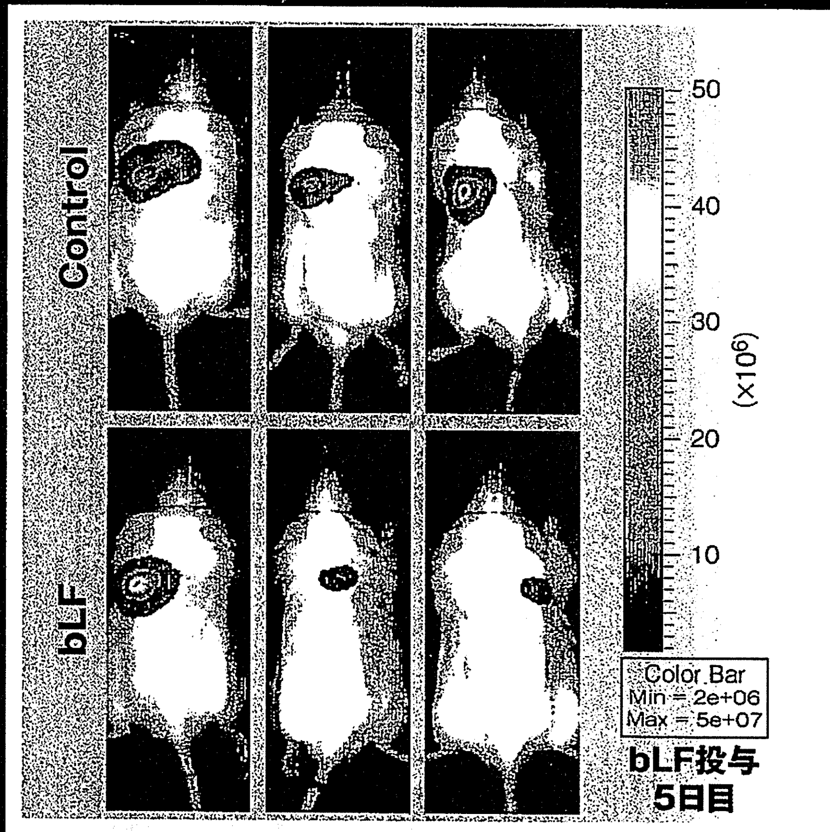
Hirohiko Hirano	The degree of masseter muscle tension and its relationship with chewing ability in Japanese elderly				submitted

Ⅳ.研究成果の刊行物・別刷

ラクトフェリン 2011

編集　日本ラクトフェリン学会第4回学術集会実行委員会

ラクトフェリン研究の新たな発展を期して



日本医学館

ラクトフェリン＋ラクトパーオキシダーゼ配合錠菓の口臭抑制効果と唾液中細菌に対する影響

ラクトフェリン 2011

八重垣 健[1)]，新 光一郎[2)]，田中とも子[1)]，
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Effects of bovine lactoferrin and lactoperoxidase-containing tablets on oral malodor and salivary bacteria

口臭の主要原因物質は，硫化水素(H_2S)，メチルメルカプタン(CH_3SH)などの揮発性硫黄化合物(VSC)である．一方，ラクトフェリン(LF)とラクトパーオキシダーゼ(LPO)は，唾液にも存在し口腔保健向上に寄与すると考えられている．そこで，ウシLF(100 mg/錠)とLPO(1.8 mg/錠)を配合した錠菓(森永乳業製)の口臭抑制効果と唾液中細菌への影響を，ランダム化二重盲検プラセボ対照クロスオーバー臨床試験で検討した．

LF＋LPO配合錠菓2錠またはプラセボ2錠を約1時間の間隔で摂取させ，摂取前後の口腔内空気のVSC濃度をガスクロマトグラフィーで測定した．その結果，プラセボ群にくらべて実験(LF＋LPO)群では，1錠目摂取直後において，CH_3SHと総VSC濃度が有意に低下した．また2時間後のH_2Sの中央値，および1錠目摂取直後から2時間後のCH_3SH中央値が嗅覚閾値を下回った．唾液中細菌叢のT-RFLP解析では，実験群で有意に低下したフラグメントが1種類検出され，*Prevotella*，*Porphyromonas*，*Treponema*などの歯周病菌の類縁菌と推定された．

以上の結果から，LF＋LPO配合錠菓による口臭抑制効果が確認され，口腔内細菌の抑制が示唆された．

Ken Yaegaki[*1)], *Kouichirou Shin*[*2)], *Tomoko Tanaka*[*1)],
Koji Yamauchi・*Tomohiro Toida*・*Keiji Iwatsuki*[*2)]

Key words：ラクトフェリン，ラクトパーオキシダーゼ，口臭，唾液，細菌

口臭の主な原因物質は，揮発性硫化物(VSC：volatile sulfur compounds)である．口臭の原因となるVSCには，硫化水素(H_2S)，メチルメルカプタン(CH_3SH)，ジメチルサルファイドがある．ジメチルサルファイドは濃度もきわめて低く，いくつかの全身疾患で上昇する[1)]．また，低濃度では"焼き海苔"の臭いに近く，必ずしも悪臭ではない．そのため，口腔由来の口臭の臨床研究では，H_2SとCH_3SHのみの測定が行われている[2)]．以上のVSCは悪臭が強いばかりか，毒性がきわめて強い事実も口臭予防の必要性の大きな

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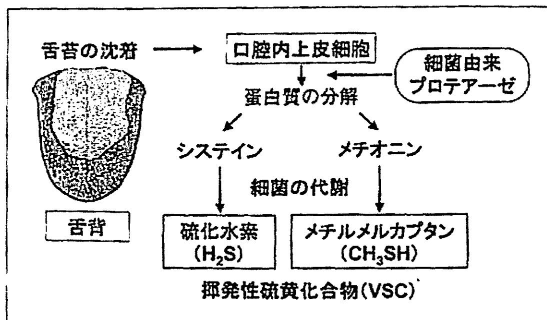

図1　口臭産生経路
VSC産生は細菌由来のプロテアーゼによる舌苔などの蛋白質の分解にはじまる．システインから硫化水素が，メチオニンからメチルメルカプタンが産生される．

根拠となる．

VSCでは，死亡事故が発生し，近年では硫化水素による自殺も起きている．VSCの毒性は青酸ガスの毒性に類似する．すなわち，ミトコンドリア・チトクロームオキシダーゼのFe^{3+}に結合し，呼吸鎖を阻害して細胞内窒息を生じる．通常の口臭濃度0.3 ppmでも悪心，呼吸障害などが発生し，口臭の1/5以下の20 ppb程度でも，眼・呼吸器・中枢神経症状があらわれる[3]．

口腔内では，VSCによる種々の毒性が報告されている．歯周炎（歯槽膿漏）の発生機序について，免疫学，細菌学あるいは生化学の面から種々の病因がある．それらが複雑に関連しあって歯周炎は発生する．

ところが，多くの病因論があっても一致するのは，歯肉上皮のバリアー効果である．このバリアーがいったん損傷を受けると，歯肉上皮の透過性が上がり，上記の病因論に基づく歯周炎病態発生過程が促進することになる．また歯肉粘膜の透過性は発がんにも関わる．

カナダがん協会は口腔がん危険性の自己診断に使用する“自己診断アンケート”のなかで，かなりの比重を持って“アルコールを含む洗口剤を毎日使いますか？”と質問している．アルコールの脱水作用で粘膜の透過性が亢進し，発がん物質の透過が増えDNA損傷が増加し，発がん性が増すとの理由である．

ところがVSCは，口腔粘膜の透過性を大きく亢進させることが判明している[4]．PGE_2などの起炎因子や菌体内毒素が容易に浸透する．一方，歯肉溝（歯と歯肉がつくる溝，歯周炎が発生する部位）には高濃度のVSCが存在し，VSCは歯周炎の原因の一つと考えられるようになった[5]．筆者らの研究では，歯肉コラーゲン合成の阻害，コラーゲン分解の亢進，骨芽細胞の増殖抑制，歯周組織の各種細胞のアポトーシス増加など，さまざまな歯周病原性が確認されている[6~15]．

口腔内でのVSC産生機序を図1に示した．脱落上皮細胞からなる舌苔を蛋白源として，含硫アミノ酸から最終的には産生されるが，細菌由来プロテアーゼによる口腔内蛋白質分解によりVSC産生ははじまる．したがって，細菌のコントロールが口臭予防の重要な手段となる．そのため洗口剤や歯磨剤中の殺菌成分によるコントロールが，現在では主体である．消臭効果を期待した食品として，チューインガムや錠菓などがあるが，食品による細菌コントロール製品は，いまだ商品化されていない．食品による口臭抑制は，使用する場所や時間帯の柔軟性にすぐれており，細菌コントロール製品の開発が期待されている．

一方，ラクトフェリン(LF)とラクトパーオキシダーゼ(LPO)は，唾液や乳などの分泌液に含有され，抗菌作用があることから，口腔内では口腔保健の維持に働くことが考えられる[16~18]．また，歯周炎予防効果も期待されている[19]．

そこで最近，筆者らはウシLFとLPOを配合した錠菓（LF＋LPO配合錠菓，森永乳業製，LF 100 mg＋LPO 1.8 mg/錠）を作製し，その口臭抑制効果を判定した[20]．本稿では，LF＋LPO配合錠菓の口臭予防効果について，細菌学的観察を含め概説する．

VSCの減少

筆者らは，VSCの減少をランダム化二重盲検プラセボ対照クロスオーバー臨床試験で検討した．口臭測定にはガスクロマトグラフィーを使った．すなわち，炎光光度検出器にSフィルターを装着した島津GC8APFpガスクロマトグラフィーを用いた．ガスクロマトグラフィーの試料注

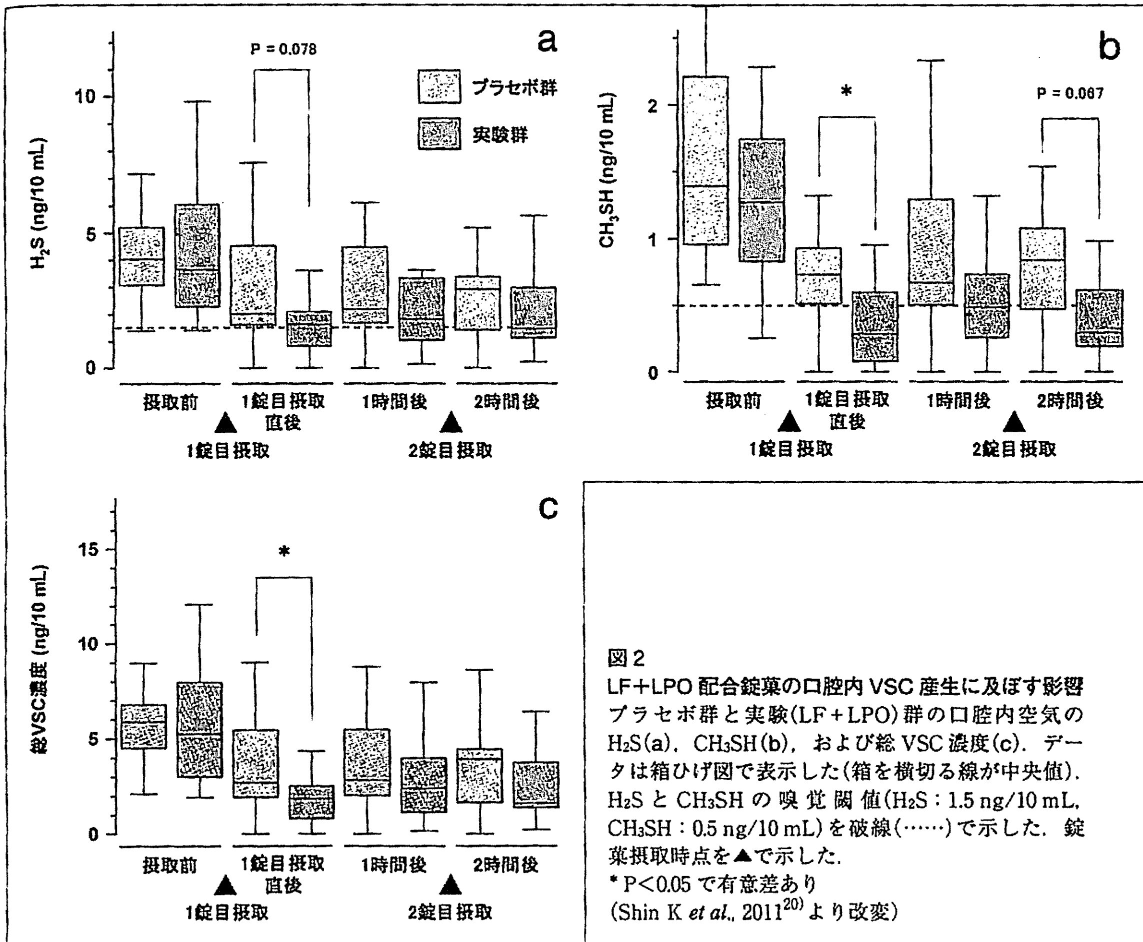

図2
LF+LPO配合錠菓の口腔内VSC産生に及ぼす影響
プラセボ群と実験(LF+LPO)群の口腔内空気の H_2S(a)，CH_3SH(b)，および総VSC濃度(c)．データは箱ひげ図で表示した(箱を横切る線が中央値)．H_2S と CH_3SH の嗅覚閾値(H_2S：1.5 ng/10 mL，CH_3SH：0.5 ng/10 mL)を破線(……)で示した．錠菓摂取時点を▲で示した．
*P<0.05で有意差あり
(Shin K *et al.*, 2011[20]より改変)

入口に10 mLの口腔内空気を注入すると，大きな逆圧が発生し，正確な測定が出来ない．そこで，6-portバルブに10 mLのサンプルループを取り付け，いったんサンプルループ内に採取した10 mLの試料をバルブ操作でキャリアガスに乗せ，カラムに注入した．カラムには，テフロンカラム1，2，3-TCEP 25% Shimalite 80-100 mesh，AW-DMCS処理，3.1 m×3.2 mm(島津製作所)を用いた．口腔内空気のVSC測定方法は既報に従った[21]．

対象者はガスクロマトグラフィーで嗅覚閾値(H_2S>1.5 ng/10 mLまたはCH_3SH>0.5 ng/10 mL)を超える口臭のあるボランティア15名とし，食品の日常的な摂取を想定して各クロスオーバー期においてLF+LPO配合錠菓2錠またはプラセボ錠を約1時間の間隔で摂取させた．摂取前，1錠目摂取直後，1時間後(2錠目摂取直前)，2時間後の時点で口腔内空気のVSC濃度を測定した．その結果を図2に示す[20]．プラセボ群にくらべて実験(LF+LPO)群では，1錠目摂取直後において，CH_3SHと総VSC濃度が有意に低下した(図2bc)．プラセボ群では各時点でH_2SおよびCH_3SHの中央値が嗅覚閾値を上回ったのに対し，実験群では2時間後のH_2Sの中央値および1錠目摂取直後から2時間後のCH_3SHの中央値が嗅覚閾値を下回った(図2ab)．

これらの結果から，LF+LPO配合錠菓による摂取開始後2時間内での口臭抑制効果が確認された．

表1　唾液中細菌数の培養法および定量 PCR 法による測定

測定項目	群	摂取前	2時間後
培養法(齲蝕関連菌数)		生菌数(log_{10} CFU/スワブ±SE)	
乳酸桿菌数	プラセボ群	2.76±0.06	2.74±0.04
	実験群	2.77±0.05	2.75±0.04
総レンサ球菌数	プラセボ群	5.75±0.22	5.53±0.20
	実験群	5.79±0.25	5.78±0.21
ミュータンス菌数	プラセボ群	2.97±0.15	2.84±0.09
	実験群	2.86±0.09	2.84±0.09
定量 PCR 法(歯周病関連菌数，総菌数)		コピー数(log_{10} コピー/10 µL±SE)	
A. actinomycetemcomitans	プラセボ群	1.11±0.08	1.16±0.10
	実験群	1.10±0.07	1.16±0.12
P. gingivalis	プラセボ群	1.49±0.27	1.57±0.31
	実験群	1.47±0.25	1.52±0.28
P. intermedia	プラセボ群	1.65±0.28	1.61±0.25
	実験群	1.72±0.30	1.64±0.27
F. nucleatum	プラセボ群	5.13±0.28	5.06±0.27
	実験群	4.96±0.35	4.92±0.28
主な口腔内総菌数	プラセボ群	7.46±0.18	7.31±0.15
	実験群	7.38±0.22	7.25±0.17

平均±SE　(Shin K *et al.*, 2011[20] より改変)

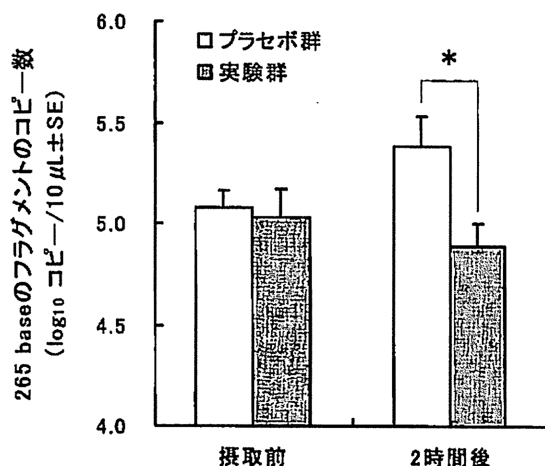

図3　唾液中細菌叢の T-RFLP 解析
唾液サンプルから精製した DNA を鋳型として蛍光ラベルプライマーを用いた PCR を行い，制限酵素(*Hae* Ⅲ)処理後，DNA シーケンサーで解析した．265 base のフラグメントは，2時間後においてプラセボ群にくらべて実験(LF+LPO)群で有意に低下した．
平均±SE．* $P<0.05$ で有意差あり
(Shin K *et al.*, 2011[20] より改変)

唾液中細菌叢への影響

錠菓摂取前および2時間後の時点で安静時唾液2 mL を採取し，細菌学的分析を行った．培養法と定量的 PCR 法で検出・測定した結果を表1に示す．測定したすべての細菌において，実験群とプラセボ群の間で差はなく，摂取前と摂取後2時間の間でも差がみられなかった[20]．一方，唾液中細菌叢の T-RFLP(terminal restriction fragment length polymorphism)解析では，2時間後においてプラセボ群にくらべて実験群で有意に低下したフラグメントが1種類検出された(図3)．この 265 base のフラグメントは，データベース検索により *Prevotella*, *Porphyromonas*, *Treponema* などの歯周病菌の類縁菌と一致した[20]．

これら細菌は VSC 産生能の高い歯周病原菌に一致しており，LF+LPO 配合錠菓による摂取開始後2時間内での VSC 産生細菌の抑制が示唆された．これらの結果は，LF および LPO による歯周病原菌の抑制に関する他の研究結果に近似する[19,22〜24]．

結　語

LF+LPO 配合錠菓には口臭抑制効果が確認され，口臭産生菌の抑制が示唆された．すなわち，細菌のコントロールを介した新たな口臭治療法と

してのLF+LPO配合錠菓の可能性が示唆された．また，口臭臨床研究での細菌学的観察におけるT-RFLP解析の有用性が確認された．

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Volume 8

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Brain Cancer

The 2007 WHO classification is updated. Brain Tumor angiogenesis, grading, heterogeneity, diagnosis, and therapies are explained in detail. Gliomas, especially the glioblastoma, the most common brain cancer, are discussed extensively, including the identification of biomarkers and stem cells. The importance of the role of immunohistochemistry in diagnosing brain tumors is emphasized. Pediatric tumors and classification, symptom, and MRI of spinal cord tumors are explained.

This eighth volume in the series **Methods of Cancer Diagnosis, Therapy, and Prognosis** discusses in detail the classification of the CNS tumors as well as brain tumor imaging in Europe. Scientists and Clinicians have contributed state of the art chapters on their respective areas of expertise, providing the reader a whole field view of the CNS tumors and brain tumor imaging.

This fully illustrated volume

- Explains the genetics of malignant brain tumors and gene amplification using quantitative-PCR
- Presents a large number of standard and new imaging modalities, including magnetic resonance imaging, functional magnetic resonance imaging, diffusion tensor imaging, amide proton transfer imaging, positron emission tomography, single photon emission computed tomography, magnetic resonance single voxel spectroscopy and intraoperative ultrasound imaging, for staging and diagnosing various primary and secondary brain cancers.
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- Includes the subject of translational medicine.

Professor Hayat has summarized the problems associated with the complexities of research publications and has been successful in editing a must-read volume for oncologists, cancer researchers, medical teachers and students of cancer biology.

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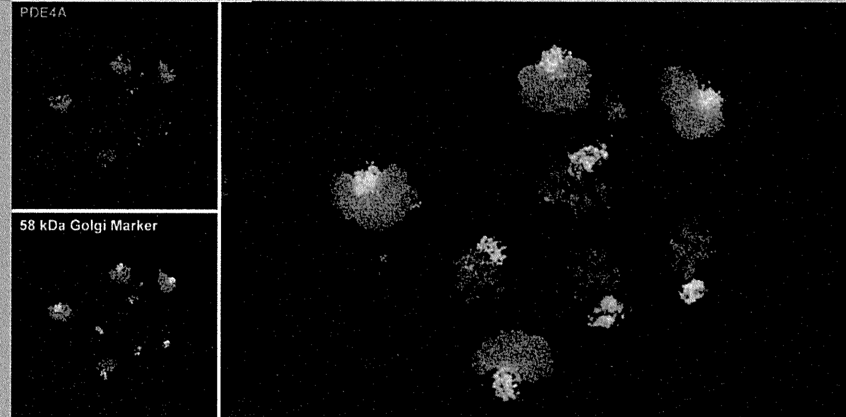
Methods of Cancer Diagnosis, Therapy, and Prognosis

Methods of Cancer Diagnosis, Therapy, and Prognosis

Volume 8
Brain Cancer



M.A. Hayat
Editor

Chapter 37

Induction of Apoptosis in Human Keratinocyte Stem Cells: The Role of Hydrogen Sulfide

Ken Yaegaki, Bogdan Calenic, and Toshio Imai

Abstract Apoptosis of epithelial stem cells is currently attracting much attention for the role it could play in cancer development. Because deficiencies in apoptosis mechanisms may be one of the causes of neoplasm formation, targeting cancer stem cells for treatment with keratinocyte stem cells could produce lasting results. However, some aspects of the apoptosis of human keratinocyte stem cells are not well understood. In this chapter, we clarify the relationship between hydrogen sulfide (H_2S) and keratinocyte stem cells. H_2S is produced in many human tissues and has been shown to cause apoptosis in the cells of epithelial tissue components. In our studies, the percentage of apoptotic cells in a population of keratinocyte stem cells was significantly increased following H_2S exposure. The mitochondrial membrane was depolarized, and ROS levels were significantly increased because of both cytochrome c oxidase and superoxide dismutase inhibition by H_2S. As a result, mitochondrial apoptotic pathways, including cytochrome c and caspase-9 and -3, were activated, while caspase-8 remained inactive. The p53 and BAX levels also were significantly elevated. We concluded that H_2S induces apoptosis in human keratinocyte stem cells through intrinsic pathways. Further, we developed a novel protocol for separating keratinocyte stem cells from human mucosa using magnetic separation, by means of which we found that the $\alpha_6\beta_4$-integrin-positive, CD71-negative fraction was also the keratinocyte stem-cell fraction.

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Keywords Apoptosis · Keratinocyte stem cells · Hydrogen sulfide · Lethal · Collagen · Carcinogenesis

Introduction

Hydrogen sulfide (H_2S) is a malodorous and lethal compound that is produced in the intestine, the oral cavity, and many other tissues at a concentration of 1 to 5 μg/g of tissue (Wiliński et al., 2011). As a transmitter or modulator of many physiologic processes, H_2S plays an important role in the cardiovascular system, the gastrointestinal tract, and the central nervous system (Zhao and Wang, 2002; Abe and Kimura, 1996; Fiorucci et al., 2006). However, its pathologic activity and toxicity to human tissues deserve greater attention. Low concentrations of H_2S exhibit several toxicities. H_2S and methyl mercaptan (CH_3SH), which is similar to H_2S, combine with collagen; when they are incorporated directly into peptide chains (Johnson and Tonzetich, 1985; Johnson et al., 1992), the intermolecular cross-linkages of collagen are cleaved (Johnson et al., 1985). Yaegaki et al. (1986) assessed the effect of CH_3SH on the intra- and extracellular metabolism of collagen in human fibroblasts and found that collagen synthesis was suppressed by approximately 40% and the intracellular degradation of newly synthesized collagen was increased (Johnson et al., 1996). Moreover, the authors have recently reported that H_2S induces apoptosis, which may be associated with carcinogenesis or its treatment, in several tissues (Yaegaki et al., 2008; Irie et al., 2009, Calenic et al., 2010a, b, Kobayashi et al., 2011).

Keratinocyte stem cells participate in many important functions. One of these is providing a barrier

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against the environment, especially microorganisms and their products, in human tissues. H_2S reduces the effectiveness of epithelial tissues in providing a protective barrier against penetration and invasion by environmental chemicals, detrimental compounds, and microorganisms (Ng and Tonzetich, 1984). This malfunction may be due to H_2S-induced apoptosis. Stem cells have been shown to play an important role in both carcinogenesis and cancer treatment (Wyllie et al., 1994; Wyllie, 1997). Failure of apoptosis in cells containing damaged DNA might be one of the causes of carcinogenesis (Wyllie, 1997) and may be the result of many factors, such as oxidative stress. On the other hand, targeting cancer stem cells – that is, apoptosis – is the only way to achieve lasting cancer-treatment responses (Wyllie et al., 1994). The mechanism of apoptosis in keratinocyte stem cells has never been described. This chapter focuses on the role of H_2S in apoptosis in human keratinocyte stem cells.

General Toxicity and Concentration of H_2S

The toxicity of H_2S is comparable to that of hydrogen cyanide. Like the cyanide ion, H_2S is recognized as a potent inhibitor of cytochrome c oxidase (COX), which is a key enzyme in the mitochondrial respiratory chain producing adenosine triphosphate (ATP) (Dorman et al., 2002). H_2S binds the heme iron of COX and inhibits the enzyme. Human exposure to H_2S, which can occur in accidents or by environmental pollution at concentrations over 500 ppm, result in immediate death (Reiffenstein et al., 1992). Acute exposure of the eye to 25 ppm H_2S causes injury; with chronic exposure, serious eye damage may occur (Lambert et al., 2006). Chronic exposure to even 300 ppb H_2S causes nausea in humans. However, this concentration is much smaller than that found in human tissues, (Wiliński et al., 2011). On the other hand, free sulfide was not detected in the serum (Togawa et al., 1992), therefore such small concentration may have an adverse effect on health. H_2S at 60 ppb (0.6 ppm of sulfide) has been used in experiments examining the adverse effects of H_2S, because this simulates conditions in human tissues (Johnson and Tonzetich, 1985; Johnson et al., 1985, 1992, 1996; Yaegaki et al., 1986, 1995, 2008).

Production of Reactive Oxygen Species by Hydrogen Sulfide

Yaegaki et al. (2008) reported that H_2S inhibited the activity of superoxide dismutase (SOD) in human gingival fibroblasts as well as purified Cu, Zn-SOD and Mn-SOD activities. SOD is a critical enzyme responsible for the elimination of superoxide radicals such as O^{2-}, one of the reactive oxygen species (ROS). The increased ROS in cells is a cause of cellular oxidative stress. If SOD activity is inhibited, the stress may remain at a high level and lead to damage of the DNA. An increment of ROS was also found in fibroblasts, mucous epithelial cells (keratinocytes), and keratinocyte stem cells derived from human skin (Yaegaki et al., 2008, Calenic et al., 2010a, b). COX inhibition by H_2S is one of the causes of ROS accumulation, while blocking of the respiratory chain also leads to cell death. However, the most important roles of SOD are in anti-aging and anti-cancer activity; if cells survive under great oxidative stress, H_2S might be considered carcinogenic (Yaegaki et al., 2008).

Carcinogenesis by H_2S

There is a strong possibility that H_2S is carcinogenic in the human intestine. H_2S exists in the human colon at over 1,000 ppm, larger than the lethal dose; to protect itself, colonic epithelium controls the toxicity by methylation and de-methylation (Kanazawa et al., 1996; Levitt et al., 1999). Thiols form persulfides with sulfhydryl and thus reduce the activity of H_2S toxification, leading to H_2S accumulation and the possibility of tissue damage (Levitt et al., 1999). In fact, exposure of colonic mucosa to high concentrations of H_2S results in considerable damage, including apoptosis (Pitcher and Cummings, 1996). High concentrations of H_2S were also found in the feces of subjects who were at high risk of developing colon cancer (Kanazawa et al., 1996). Proliferation of colonic mucous cells is accelerated by H_2S through the Ras subfamily (Christl et al., 1996). Moreover Ki-Ras is activated in colorectal cancers (Bos et al., 1987). The Ras oncogene is implicated in colorectal carcinogenesis by H_2S (Deplancke and Gaskins, 2003; Picton et al., 2002; Pitcher and Cummings, 1996; Christl et al., 1996; Bos et al., 1987).

Thus, H_2S has two conflicting effects on cells – apoptosis and cell proliferation – signifying carcinogenicity (Yang and Wang, 2007). There is also an implication that defective apoptosis may permit the survival of mutated cells that could have undergone genomic changes similar or identical to those known to be expanded in cancer (Wyllie et al., 1994; Wyllie, 1997; Federici et al., 2011). In fact, H_2S also prevent from apoptosis in several tissues under specific conditions: this would be a reason of defecting apoptosis which might lead the process of carcinogenesis (Taniguchi et al., 2011).

After human fibroblasts, oral epithelial cells, and keratinocyte stem cells derived from skin were exposed to 100 ng/mL H_2S, DNA damage was identified and measured by means of single cell gel electrophoresis (SCGE) assay (CometAssy^{TM}, Trevigen, Gaithersburg, MD, USA). The basis of SCGE is the electrophoretic migration of DNA in an agarose gel. Intact DNA remains within the nucleoid whereas damaged DNA migrates away from it. Under a microscope, the nucleus together with the migrated DNA has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments. Evaluation of the comet-tail shape allows for the assessment of DNA damage: measurements include *tail length* (whole length of comet – length of head), *%DNA in the tail* (tail area/[tail area + head area] × 100), and *tail moment* (tail length × %DNA in the tail). When human fibroblasts, oral epithelial cells, and keratinocyte stem cells derived from skin were exposed to H_2S, a significant increase was found in the test groups compared to the controls in tail length and tail moment as well as in DNA in the tail (Calenic et al., 2010b). Thus, genotoxicity of H_2S has been demonstrated in the aforementioned cell lines using SCGE, and it is suggested that H_2S-induced DNA damage might be carcinogenic.

Hydrogen Sulfide–Induced Apoptosis in Human Keratinocyte Stem Cells

H_2S-induced apoptosis was demonstrated in clonal human keratinocyte stem cells derived from adult human skin (Celprogen, San Pedro, CA). These cells tested positive for the following stem-cell markers: Keratin 19, CD34, CD133, and Nestin. Although the manufacturer's instructions allowed up to 12 passages, the cells were used for only 10 passages because of the strong potential for natural differentiation to occur. The cells were incubated with H_2S at 50 ng/m; H_2S in the medium was measured at 18 ng/mL (0.5 μmol/L). Flow cytometry was used to detect the apoptotic cells (Calenic et al., 2010b), and the number of cells was found to increase in a time-dependent manner. After 24 h incubation, early apoptotic cells accounted for approximately 20% of the total cell population; after 48 h, more than 30% were found to be apoptotic. Late apoptosis or necrotic cells remained very low after incubation (Calenic et al., 2010b), and it is suggested that most of the cells were not necrotic.

Since apoptosis has been demonstrated to occur in keratinocyte stem cells at such a low concentration of H_2S, it is highly likely that the cells of many tissues, such as the esophagus, airway, the lung, the vagina, and the uterus, undergo apoptosis caused by H_2S. There are two possibilities for the biologic role of the stem cells as described above. If apoptotic function is deficient, H_2S might be one of the causes of neoplasm formation in these tissues. On the other hand, a possible treatment for tumors might focus on causing apoptosis in the stem cells of these tissues.

Pathway for Apoptosis Caused by Hydrogen Sulfide

Depolarization of the mitochondrial membrane in keratinocyte stem cells derived from adult human skin (Celprogen) was evaluated by measurement of the uptake of a cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, into the mitochondria (Fig. 37.1). Dyed cells were analyzed and counted by a flow cytometer. The percentage of mitochondrial membrane–depolarized cells was significantly increased by H_2S incubation (Calenic et al., 2010b).

Calenic et al. (2010b) determined the amount of cytochrome c released into cytosol and found that it was significantly increased by H_2S incubation. Both caspase-9 and -3 activities were significantly increased following H_2S exposure, but caspase-8 was not activated (Calenic et al., 2010b).

There are two main streams in the causes of apoptosis: the cell-death ligand/receptor pathway, including

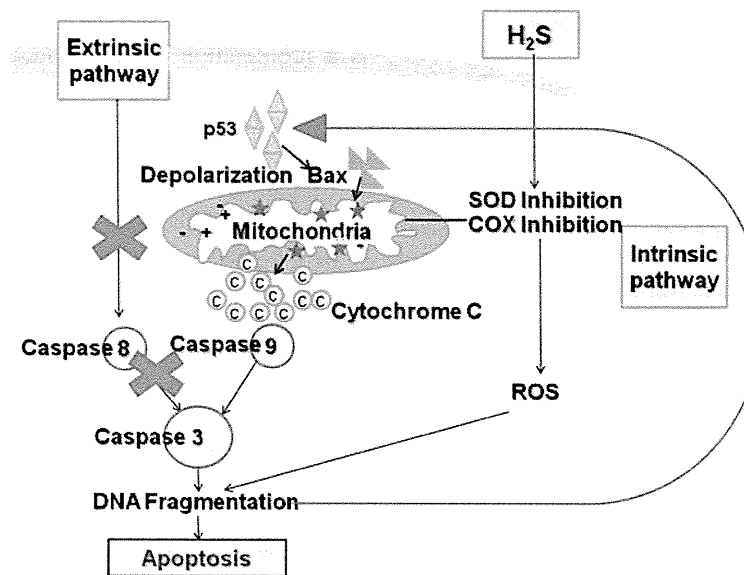

Fig. 37.1 Pathways for Apoptosis Caused by Hydrogen Sulfide in Human Keratinocyte Stem Cells. After depolarization of the respiratory chain, the release of cytochrome c into the cytosol increased, followed by caspase-9 activation. An increase in p53 then activated BAX, resulting in mitochondrial depolarization. Because H_2S strongly inhibits SOD, an increase in the amount of ROS by the inhibition of SOD activates the mitochondrial apoptotic pathway as well as COX inhibition by H_2S. Since caspase-8 was not activated in keratinocyte stem cells following H_2S exposure, it is suggested that the extrinsic pathway might not be involved in H_2S-induced apoptosis

caspase-8, and the intrinsic mitochondrial pathway. H_2S depolarizes the mitochondrial membrane potential and increases the release of cytochrome c into cytosol, followed by caspase-9 activation. These changes trigger mitochondrial-dependent apoptosis. Yaegaki et al. (1986) reported that H_2S strongly inhibits SOD as described above; an increase in the amount of ROS by the inhibition of SOD may initiate the mitochondrial apoptotic pathway as well as COX inhibition by H_2S. Caspase-3 in particular is implicated as the effector caspase, since both receptor/ligand–mediated and mitochondrial pathways unite at the level of caspase-3 activation. Thus, amplified caspase-3 activity means that apoptotic mechanisms are ongoing. It has been shown that caspase-3 activity is considerably increased by H_2S incubation and that the pathway for mitochondrial-dependent apoptosis is activated by H_2S (Calenic et al., 2010b).

Caspase-8, which is known to be an apoptotic initiator activated by the cell-death receptor/ligand–mediated apoptotic pathway, also promotes the release of cytochrome c through cleavage of pro-apoptotic Bcl-2 family members. Since caspase-8 was not activated in keratinocyte stem cells following H_2S exposure, it would seem that the extrinsic pathway might not be involved in H_2S-induced apoptosis (Calenic et al., 2010b).

Role of p53 and Bax Apoptosis Caused by Hydrogen Sulfide

After H_2S exposure, p53 and phosphorylated p53 at serine were both found to be significantly increased in human keratinocyte stem cells (Fig. 37.1). Bax levels also increased in a time-dependent manner. In the mitochondrial pathway, Bax, a pro-apoptotic member of the Bcl-2 family, is a p53 primary-response molecule which also mediates mitochondrial membrane depolarization, activating the mitochondrial apoptotic pathway. On the other hand, p53 triggers both mitochondrial pathways and cell-death ligand/receptor pathways involving caspase-8 (Hama-Inaba et al., 2001). However, p53 may not trigger the extrinsic pathway in H_2S-induced apoptosis of keratinocyte stem cells, as demonstrated above (Calenic et al., 2010b).

Separation of Keratinocyte Stem Cells from Human Mucosa

In human epithelia, the basal epidermal layer is composed of three distinct types of cells: keratinocytes, keratinocyte stem cells, and transit amplifying cells (Fig. 37.2). Keratinocyte stem cells play an important role in the physiologic processes of protecting not only tissues but also individuals from harmful environmental factors. In other words, keratinocyte stem cells may have key responsibilities in tissue or individual homeostasis. Hence, we carried out the research described above using cloned stem cells obtained from human skin.

Because our interest is in the cells of the internal organs, keratinocyte stem cells derived from human mucous membrane should be used in our studies. However, we have found it extremely difficult to isolate cells from human mucous membrane. We have tried to separate and characterize keratinocyte stem cells derived from human oral mucosa, because it is easy to reach.

Basal cells, including stem cells, adhere to the basement membrane via integrins, which are adhesion molecules. $\alpha_6\beta_4$ integrin is expressed only on the surface of basal keratinocytes, including both keratinocyte stem cells and transit amplifying cells; however, transit amplifying cells express CD71, which is a proliferation-related surface marker, while quiescent cells such as stem cells show extremely low expression of CD71. Therefore, Calenic et al. (2010c) developed separation procedures using a magnetic cell-separation system employing antibodies to these markers conjugated with magnetic microbeads.

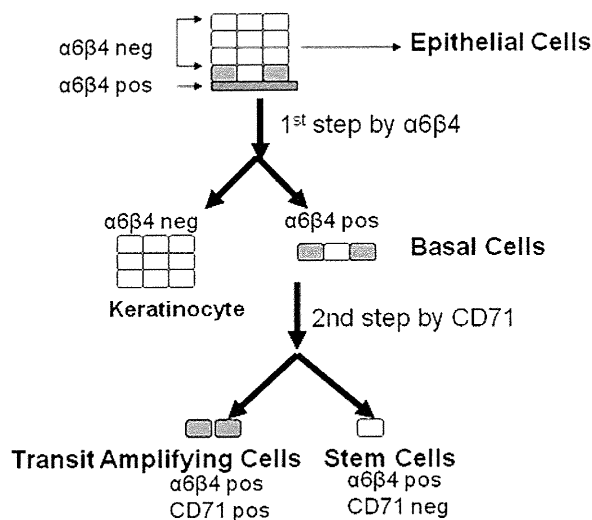

Fig. 37.2 Separation of Keratinocyte Stem Cells from Human Mucosa. In the first step, using $\alpha_6\beta_4$ magnetic separation, an $\alpha_6\beta_4$ positive cell fraction was separated. In the second step, using CD71 magnetic separation, the $\alpha_6\beta_4$ ^{pos} CD71^{neg} fraction containing stem cells was obtained

In the first step, using $\alpha_6\beta_4$ magnetic separation, we successfully separated the $\alpha_6\beta_4$ positive cell fraction, but this fraction formed only 8% of the entire cell population. In the second step, using CD71 magnetic separation, the $\alpha_6\beta_4$ ^{pos} CD71^{neg} fraction was found to contain more resting cells (G_0/G_1 phase) than the $\alpha_6\beta_4$ ^{pos} CD71^{pos} cell fraction. This fraction showed greater colony-forming capacity than the $\alpha_6\beta_4$ ^{pos} CD71^{pos} fraction (Calenic et al., 2010c). Furthermore, this fraction showed a much smaller cell size than others. Since stem cells are much smaller than transit amplifying cells, this result supports our hypothesis that the $\alpha_6\beta_4$ ^{pos} CD71^{neg} fraction is composed mostly of stem cells (Calenic et al., 2010c). Moreover, $\alpha_6\beta_4$ ^{pos} CD71^{neg} cells were very positive to p63 and keratin 19; $\alpha_6\beta_4$ ^{pos} CD71^{pos} cells were positive to keratin 10 and 19 but negative to p63; and $\alpha_6\beta_4$ ^{neg} cells were strongly positive to keratin 10 and involucrin (Calenic et al., 2010c). Because involucrin and keratin 10 are keratinocyte differentiation markers, and both p63 and keratin 19 are keratinocyte stem-cell markers, this fraction proved to be very rich in keratinocyte stem cells derived from human mucous membrane. We have found that it is possible to culture several passages of this fraction while retaining their properties as stem cells. Further studies using this fraction are in progress.

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