cell death is activation of the caspase cascade. Activation of caspase 3 is required for internucleosomal DNA degradation (Woo et al., 1998), and caspase inhibition prevents the release of apoptotic bodies from cells (Zhang et al., 1999). In the present study, supplementation of the medium with rhGas6 prevented Pi-induced caspase 3 activation. These results clearly show that Pi downregulates Gas6-Axl, decreases PI3K-mediated Akt phosphorylation, inactivates Bcl2, activates Bad, and activates caspase 3, leading to apoptosis.

The present study demonstrated that statins restored the Gas6-mediated survival pathway. Consistent with these results, Akt phosphorylation has been reported to be an antiapoptotic mechanism of statins: pravastatin inhibited hypoxia-induced apoptosis through activation of Akt in cardiomyocytes (Bergmann et al., 2004), and simvastatin and pravastatin enhanced phosphorylation of Akt and promoted angiogenesis in endothelial cells (Kureishi et al., 2000). Recently, it was reported that statins inhibit caspase 3 activation driven by protein kinase C inhibitors in the process of apoptosis, suggesting that caspase 3 is also under the control of statins during apoptosis (Tanaka et al., 2004).

In this study, we performed experiments under both short-term (within 24 h) and long-term (up to 10 days) conditions. In general, short-term experiments are able to examine acute cell behavior, such as signaling and transcription. However, because obvious HASMC calcification takes at least 3 days, we also performed long-term experiments. Downregulation of Gas6, Axl expression and reduced phosphorylation of Akt, Bcl2, and Bad, and a beneficial effect of statins were consistently found in the long-term condition. This confirms that the Gas6-Axl survival signal is the key mechanism for Pi-induced calcification.

It is concluded that statins inhibit Pi-induced apoptosis via the Gas6/Axl-PI3K-Akt signal pathway, which has a crucial role in the prevention of HASMC calcification. This study adds further evidence of the pleiotropic effects of statins, suggesting a therapeutic strategy for the prevention of vascular calcification.

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CASE REPORT

Improved cognitive function, mood and brain blood flow in single photon emission computed tomography following individual reminiscence therapy in an elderly patient with Alzheimer's disease

Katsuaki Tanaka, Yukiko Yamada, Yoshio Kobayashi, Kazuki Sonohara, Ayako Machida, Ryuhei Nakai, Koichi Kozaki and Kenji Toba

Department of Geriatric Medicine, Kyorin University, School of Medicine, Mitaka, Tokyo, Japan

An 88-year-old man who was suffering from chronic renal failure and hypertension visited our memory clinic because of recent cognitive decline and a gradual decrease in his vitality and volition. His Mini-Mental State Examination (MMSE) score was 22, his 15-item Geriatric Depression Scale (GDS-15) score was 10, and his Vitality Index (VI; full score, 10) was 6. We diagnosed Alzheimer's disease with depressive mood, and this was supported by findings of global brain atrophy by magnetic resonance imaging and decrease in brain blood flow in the posterior cingulated gyrus and frontal association area by single photon emission computed tomography (SPECT). After completion of a life review of the patient, individual reminiscence therapy was performed once a week for 2 months. After the therapy, a comprehensive geriatric assessment showed that cognitive function, depressive mood and decreased vitality had all markedly improved (MMSE, 29; GDS, 7; VI, 9). Moreover, SPECT showed improved brain blood flow, especially in the frontal lobe. We believe that this is the first case in which reminiscence therapy alone not only improved cognitive function and mood but also reduced neuroimaging abnormalities.

Keywords: Alzheimer's disease, cognitive function, life review, reminiscence, single photon emission computed tomography easy Z-score imaging system (SPECT eZIS).

Introduction

Reminiscence is a psychophysiological therapy proposed by an American geriatric psychiatrist, Robert N. Butler, in 1963,[1] and recommended as a grade D′ psychological approach to the management of neuropsychiatric symptoms of dementia by Livingstone *et al.*[2] There are two methods of reminiscence therapy: group reminiscence and individual reminiscence. The former is very common and widely performed in public welfare facilities;[3] in contrast, very few facilities perform individual reminiscence[4] and related documents and references are limited in Japan.[5,6]

Herein, we report the case of an 88-year-old man who was treated by individual reminiscence therapy at our outpatient memory clinic. We show that cognitive function, depressive condition and volition were all

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Correspondence: Kenji Toba MD, Department of Geriatric Medicine, Kyorin University, School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan. Email: toba@kyorin-u.ac.jp

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improved in a comprehensive geriatric assessment performed after therapy. Objective changes supporting these outcomes were noted in imaging after completion of the individual reminiscence program.

Case report

The patient was an 88-year-old man suffering from chronic renal failure, hypertension and hyperuricemia. He was taking Nifedipine CR tablet 20 mg/day and allopurinol tablet 50 mg/day. He had been born in Asakusa, Tokyo, and had lived at his mother's home in Gifu Prefecture while he was a primary schoolboy. His surviving family members were his wife, a second son and his wife, two grandsons and one granddaughter. His occupation had been as a private primary school teacher, and after retirement he was engaged in editing and publishing biographies of great persons at an educational book publishing company.

Based on a family interview, the patient had shown temporal disorientation, derangement of the capacity to register and decreased activities of daily living (ADL) for several years; for these reasons he visited our outpatient memory clinic. His present illness was not specific, his neurological findings were normal, and no other psychological or psychiatric symptoms were noted. In radiological imaging, global cerebral atrophy was noted in brain magnetic resonance imaging (MRI), and relative decreases in blood flow in the posterior cingulated gyrus and right median plane of the frontal lobe were noted on single photon emission computed tomography (SPECT; ethylene cystine dimer [ECD]).

Although his Geriatric Depression Scale score suggested depression, his symptoms did not satisfy major depression criteria of the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV). Dementia with Lewy bodies (DLB) was suspected based on decreased blood flow in the occipital lobe on SPECT; however, because neurological symptoms such as parkinsonism, hallucination and visual hallucination were absent, and reduction of cognitive function was mild, Alzheimer's-type dementia was diagnosed. Based on this diagnosis, administration of donepezil hydrochloride (Aricept) was considered, but the patient had chronic renal failure and we were concerned about the risk of donepezil-induced rhabdomyolysis. Considering the risk, the family requested that the patient should not receive this drug, so individual reminiscence therapy was initiated instead.

The individual reminiscence procedure was performed as follows:

1 The person with the main responsibility for taking care of the patient (the wife of his second son) first visited the hospital alone, and were interviewed about the patient's profile (shown below) before therapy. This allowed discussion of episodes that she could not mention in the presence of the patient, and allowed a prior understanding of things that should be avoided when talking to the patient. The interview clarified: (i) details of the interviewee and her relationship with the patient; (ii) the patient's diagnosis and medications; (iii) the patient's past medical history, lifestyle and other diseases; (iv) the patient's family members (i.e. marital status and presence or absence of spouse), nickname, parents, siblings, children, grandchildren, close relatives and friends, and other people; (v) native town, places of importance to the patient other than his native town, history of moving; (vi) final level of school education; (vii) professional career (i.e. thoughts on work and feelings of accomplishment); (viii) hobbies (including interests and matters of concern); (ix) likes and dislikes; (x) religion and beliefs; and (xi) particulars regarding medical care and mental state.
2 The above items were discussed in the interview, and the first session of reminiscence therapy was performed 1 week later. Subsequently, sessions of approximately 1 h were performed weekly for 8 weeks (one set of therapy).
3 The person taking care of the patient attended all the sessions to correct paramnesia, and to insert words related to the date and weather to correct temporal disorientation.
4 In the first session, the patient was allowed to talk freely about the times that he had held strong feelings. Later sessions progressed through a chronological life review from boyhood to late middle age, including background, life and manners, and social conditions of the times.
5 The following items were used to jog the memory: a cup and ball, ohajiki (small discs of glass), menko (cardboard), a five-bead abacus, an antique watch, an empty lemonade bottle, primary and junior high school text books from approximately 1910 until the mid-1920s, and a collection of old photographs from the mid-1920s until approximately 1960.
6 Before the fifth session, the effect of the first four sessions was evaluated using a Comprehensive Geriatric Assessment (CGA). The CGA was also performed after completion of all sessions before initiation of the next outpatient treatment, and the final outcome of the individual reminiscence therapy was determined. Only the Mini-Mental State Examination (MMSE), Hasegawa's Dementia Scale – Revised (HDS-R), 15-item Geriatric Depression Scale (GDS-15) and Vitality Index (VI) were evaluated after completion of the fourth session. The CGA was comprised of: (i) Barthel Index (BI) as an index of ADL (full score, 100 points); (ii) MMSE and HDS-R for evaluation of cognitive function; (iii) Dementia Behavior Disturbance Scale (DBD) for behavioral and psychological symptoms of dementia; (iv) GDS-15 as

an index of depression (full score, 15 points); (v) VI for evaluation of vitality and volition (full score, 10 points); and (vi) Zarit's Burden Interview (ZBI) for evaluation of carer's load (full score, 88 points).

There are two approaches to reminiscence therapy: one in which the patient recalls memories from historical news and events, old articles regarding every-day issues, old toys, and printed material such as old books; and a second in which a life review is held, in which the patient looks back on their own history and life and compares this with their current condition. In our case, individual reminiscence therapy was performed using the latter procedure, but the former procedure was employed concomitantly as needed for introductory purposes in the first session and as idle talk when the conversation halted during a session.

On MRI, the entire brain was seen to be markedly atrophied, and some ischemic lesions, such as periventricular high intensity lesions, were also noted. Almost no changes were noted on MRI performed 6 months after completion of the reminiscence program.

On SPECT (^{99m}Tc-ECD) Relative reduction of blood flow was noted in the frontal and occipital lobes, posterior cingulated gyrus and precuneus in easy Z-score imaging system (eZIS) analysis. In eZIS images after completion of the program, improvements were noted in regions that had previously shown reduced blood flow, with a particularly marked increase in blood flow in the frontal lobe, compared to that before therapy (Fig. 1).

Results of the CGA are given in Table 1 and as follows. At the first examination, the MMSE and HDS-R scores were 22 and 14, respectively. These scores increased to 25 and 24, respectively, after completion of four sessions of reminiscence therapy, and to 29 and 21, respectively, after completion of the program, showing a marked improvement of cognitive function compared to that before therapy. The patient showed temporal disorientation and delayed recall of three words, and was unable to enumerate a list of 10 vegetables before therapy, and these characteristics were also markedly improved by the therapy. The MMSE score was still 29 on re-evaluation 6 months after completion of the program.

The GDS-15 score was 10 on the first examination and showed no change after four sessions; however, the score decreased to 7 at completion of all sessions, indicating a slight improvement of depression, and we saw the patient smile more often than before treatment.

The VI and BI scores were 6 and 85, respectively, at the first examination, and increased to 10 and 90, respectively, after four sessions. After completion of all sessions, these scores were 9 and 95, respectively. The patient started to do things that he previously left to others, and started to read ancient documents again. The VI and BI scores remained at 9 and 95, respectively, in tests 6 months after completion of all sessions.

For the DBD and ZBI, the patient had no behavioral or psychological symptoms of dementia, and no numerical changes were noted after the therapy.

Discussion

Treatment with individual reminiscence therapy alone markedly improved attention, volition and depression in the patient. According to Butler, the pioneer of reminiscence therapy, life review is a healthy psychological behavior in which past events are re-evaluated, and this process brings about improvements in physical as well as mental and social activities,[7] thereby showing an effect on volition. Bohlmeijer *et al.* also reported that reminiscence was effective for senile depression;[8] however, there have been no reports of an objective effect on cognitive function. Several Japanese studies have suggested that reminiscence is effective mainly for psychological depressive tendency and decreased volition, but a marked effect on cognitive function has only been noted in a few reports. Kurokawa *et al.* reported that reminiscence was more effective for vascular dementia than for Alzheimer's-type dementia with regard to improvement of cognitive function,[9] and Urabe *et al.* showed that individual reminiscence improved cognitive function only in a few patients with Alzheimer's-type dementia.[5]

What was the cause of the marked improvement in cognitive function in our patient? One characteristic of the patient was an interest in ancient documents, history, education, politics and economics. He remembered some details of interesting events in his childhood and adolescence, and his memories became clearer as the sessions progressed. Furthermore, his family very cooperatively[10] attended all sessions, understood his condition in detail at each time point, and provided information to the physician, as reflected by the abundant information in the personal chart (life review) obtained before therapy. This background suggests that the following factors contributed to the effectiveness of individual reminiscence therapy for this patient: (i) a personal chart that provided extensive information prior to therapy; (ii) cooperation of the patient's family, not only in taking care of the patient but also in visiting the hospital and attending the therapy sessions; (iii) the patient's retention of memories of childhood and adolescence; (iv) the patient's interest in certain fields, although not very active; and (v) the patient had been solitary, and the sessions provided company and the chance for conversation. The effectiveness of individual reminiscence therapy may be greater in cases that follow this pattern.

An important characteristic of this case was the increased blood flow in the frontal association area, which is considered to be the center of volition, in

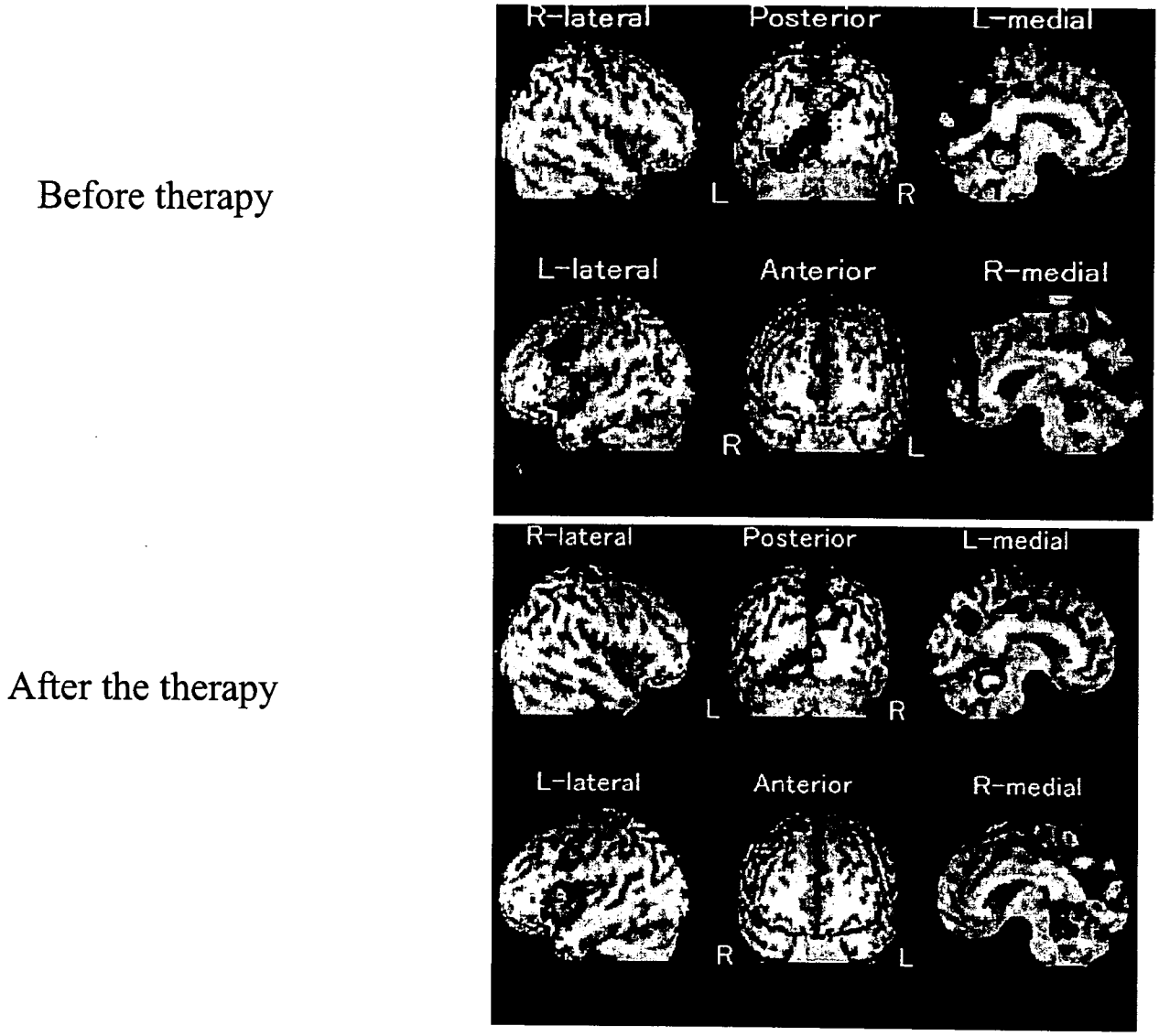

Figure 1 Easy Z-score imaging system (eZIS) image of single photon emission computed tomography (SPECT) before and after individual reminiscence therapy. Reduction of blood flow in the right frontal, and both left temporal, median-parietal and occipital lobes, posterior cingulated gyrus, and precuneus (upper panel). Comparing the SPECT findings before therapy, marked increase in the frontal lobe is noted (bottom panel).

SPECT (eZIS) performed after reminiscence therapy. Ushijima *et al.* performed SPECT and MMSE in 59 patients with Alzheimer's-type dementia and in 12 normal volunteers to find areas of reduced blood flow, and investigated the relationship between cognitive function and blood flow;[11] the results showed that attentiveness and calculation ability were associated with reduced blood flow in the frontal cortex. Migneco *et al.* reported that decreased volition (apathy) in Alzheimer's disease is related to reduced blood flow in the anterior cingulated gyrus in SPECT,[12] and Holthoff *et al.* also investigated decreased volition in early stage Alzheimer's disease by positron emission tomography, and found that decreased blood flow in the left orbitofrontal region had an influence.[13] Although association of decreased cognitive function and volition with a reduction of regional blood flow in the brain has been reported,[14] there has been no previous report of a marked increase in cerebral blood flow caused by individual reminiscence therapy. Therefore, this case provides an important demonstration of the relationship between blood flow in the frontal lobe and volition and cognitive function.

Table 1 Effect of reminiscence therapy on the score of Comprehensive Geriatric Assessment

	Before 13 May	After 2 months 5 August	End of session 16 September
Barthel Index (0–100)	85	90	95
MMSE (0–30)	22	25	29
HDS-R (0–30)	14	24	21
GDS (0–15)	10	10	7
Vitality Index (0–10)	6	10	9
Zarit Burden Interview (0–88)	21		19

GDS-15, 15-item Geriatric Depression Scale; HDS-R, Hasegawa's Dementia Scale – Revised; Mini-Mental State Examination (MMSE).

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〈原　著〉

超高齢者におけるクレアチニンクリアランス推定式の比較検討

平山　俊一[1)]　菊池　令子[2)]　井上慎一郎[2)]　塚原　大輔[2)]　末光　有美[2)]
小林　義雄[2)]　杉山　陽一[2)]　長谷川　浩[2)]　神崎　恒一[2)]　井上　剛輔[3)]
鳥羽　研二[2)]

要　約　**目的**：高齢患者は外来では24時間クレアチニンクリアランスの測定が困難であり，服用薬物数も多いため，クレアチニンクリアランス実測値をできるだけ正確に反映する推定式を利用することは臨床上重要である．**対象**：各種基礎疾患を有する85歳以上の超高齢者67名を含む入院高齢者143名（男性73名　女性70名　平均年齢82.9±8.6歳）．**方法**：4種のクレアチニンクリアランス推定式から得られた推定値と24時間クレアチニンクリアランスの実測値との相関を比較検討した．**結果と結論**：全体として今回の検討では超高齢者においてもCockcroft and Gaultの式による推定値が最もよい相関を示した．85歳以上の女性超高齢者において実測値と推定式の相関が低く，推定式の改定についても今後の検討課題と思われる．

Key words：超高齢者，クレアチニンクリアランス，推定式，Cockcroft and Gaultの式，安田の式

（日老医誌　2007；44：90-94）

緒　言

高齢社会の到来により，外来入院を問わず，高齢患者が増加の一途をたどっている．厚生労働省の推計によると，2004年度において85歳以上の超高齢者は273.4万人と報告されている[1)]．高齢者に腎排泄型薬剤を投与する際，適正な用量を設定するため腎機能を正確に評価する必要がある．腎機能を表す指標として，糸球体濾過量には一般的に内因性クレアチニンクリアランス（以下Ccrと略す）が使われている．クリアランス試験には24時間蓄尿が必要であるが，時間を要することや被験者に排尿，蓄尿という負担があり繁雑であることから外来で測定することは容易ではない．このため血清クレアチニン値（以下Scrと略す）からCcrを推定するいくつかの数式が提案されている．しかしこれらの数式は実際に投薬の必要な諸疾患を有する高齢者に当てはめる際，筋肉量の減少などのためScrによるCcr推定値と実測したCcrがかけ離れた値を取ることがある．外来の超高齢患者においても適切な薬物療法を行うためには腎機能を正確に評価する必要がある．このため種々の推定式による相関を調べどの推定式が最もよく超高齢者に適合するか検討を行った．

1) S. Hirayama：東京薬科大学
2) R. Kikuchi, S. Inoue, D. Tsukahara, Y. Suemitsu, Y. Kobayashi, Y. Sugiyama, H. Hasegawa, K. Kouzaki, K. Toba：杏林大学病院高齢医学科
3) G. Inoue：都東村山老人ホーム診療所内科
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対象及び方法

杏林大学病院高齢医学科に2004年9月から2006年1月の間に入院した60歳以上の症例のうち，短期入院や，蓄尿不可能症例を除外し，尿道留置カテーテルを使用している患者や蓄尿が可能と判断された症例全例を対象にした．疾患や治療による除外は設けず，脳血管障害，感染症，経口摂取不良，利尿剤，補液などの様々な基礎疾患，治療を有する高齢者（平均年齢82.9±8.6歳（男性82.0±8.8歳　女性83.8±8.3歳））例を対象に行った．男女比及び84歳以下と85歳以上の症例数に偏りはなかった（表1）．対象高齢者全体の平均Scrは1.31±0.87mg/dlであった．身体測定，血液検査，尿検査などを測定し24時間蓄尿によるCcrを計算した．なお，Ccrは未補正のものを使用した．安田の式[2)]，Cockcroft and Gaultの式[3)]（以下C&G式と略す），折田の式[4)]，Walserの式[5)]の推定値を算出し，それぞれ推定値と実測値の相関を回帰分析，相関係数の差の検定により解析し比較検討した．さらに，層別解析として，84歳までの前期及び後期高齢者群76名と，85歳以上の超高齢者67名について男女別に層別解析を行った．

また実測値と推定式からの値との一致を箱ヒゲ図で求

表1　対象年齢分布

Age（歳）	n		
	男性	女性	全体
～84	42	34	76
85～	31	36	67
全体	73	70	143

め，値が外れ値となった症例については，患者の疾患や治療の背景，測定時の問題点について調査した．

本研究は，杏林大学高齢医学の入院に際して，CCr測定値を臨床研究に使用することを口頭で説明し同意を得て試行した．

(1) 安田の式

男性：Ccr（m*l*/min）＝（176－年齢）×体重（kg）÷（100×Scr（mg/100 m*l*））

女性：Ccr（m*l*/min）＝（158－年齢）×体重（kg）÷（100×Scr（mg/100 m*l*））

(2) Cockcroft and Gault の式

男性：Ccr（m*l*/min）＝（140－年齢）×体重（kg）÷（72×Scr（mg/100 m*l*））

女性：Ccr（m*l*/min）＝{（140－年齢）×体重（kg）÷（72×Scr（mg/100 m*l*））}×0.85

(3) 折田の式

男性：Ccr（m*l*/min）＝（－0.065×年齢－0.493×BMI＋33）÷（体重（kg）×Scr（mg/100 m*l*））×144

女性：Ccr（m*l*/min）＝（－0.052×年齢－0.202×BMI＋21）÷（体重（kg）×Scr（mg/100 m*l*））×144

(4) Walser の式

男性：Ccr（m*l*/min）＝7.57÷Scr（mM）－0.103×年齢＋0.096×体重（kg）－6.66

女性：Ccr（m*l*/min）＝6.06÷Scr（mM）－0.08×年齢＋0.08×体重（kg）－4.81

成　績

85歳未満の前期及び後期高齢者群において，安田，C&G，折田，Walser の推定値と24時間蓄尿による実測値の相関係数（r）は安田 r＝0.761，C&G r＝0.761，折田 r＝0.693，Walser r＝0.553 と安田の式，C&G 式で強い傾向があった．超高齢者群において，各々の推定式による推定値と実測値の相関係数は安田 r＝0.718，C&G r＝0.739，折田 r＝0.697，Walser r＝0.645 と，安田の式，C&G 式で相関が強い傾向があった（図1，図2）．超高齢者を男女に分け両群で各々の推定値と実測値の相関係数 r を比較したところ，男性で安田 r＝0.840，C&G r＝0.841，折田 r＝0.791，Walser r＝0.736，女性で安田 r＝0.678，C&G r＝0.690，折田 r＝0.667，Walser r＝0.582 となり，男性に強い相関傾向があり，女性の相関係数は低かった（図3，図4）．また，超高齢者群において回帰係数を比較したところ，男性で安田＝0.796，C&G＝0.988，折田＝0.577，Walser＝0.375　女性で安田＝1.088，C&G＝1.262，折田＝0.776，Walser＝0.395 となった．

図5は超高齢者を男女で比較したものである．縦軸は実測値と推定値のずれの割合を示したもの（（実測値－推定値）×100/実測値）である．折田，Walser の式では，男女共に推定値が高く評価される傾向がある．

85歳以上の超高齢者での箱ひげ図における外れ値を検討し，実測値が高値となる6例の患者背景を調べた．輸液4例，利尿剤や Ca 拮抗薬など腎血流量を増加させる薬剤4例，腎不全2例，Scr 高値2例，心不全2例，CRP 高値2例であった．また，推定値が高値となる7例の患者背景を調べた．輸液5例，蓄尿不全または蓄尿少量4例，腎不全4例，癌3例，コントロール不良の糖尿病1例，胸水貯留，腹水貯留1例，肥満1例であった．

考　察

服用薬物数が多いほど薬剤有害作用の発現率は増加する傾向にある．また，加齢によってもその傾向は増加する[6]．その原因には加齢に伴う薬物動態学的・薬力学的な変化，多剤併用による相互作用，日常生活活動度（ADL）・認知機能の低下などが考えられるが，特に重大な原因として，腎機能の低下による相対的過量投与が挙げられる．Scr による腎機能の推定にはいくつか方法があるが高齢者，特に超高齢者になると筋肉量の低下により Scr が腎機能の低下と不相応な低値を示すことがしばしば見られる．Ccr 測定上の更なる問題点として正確な蓄尿の可否がある．加齢に伴う残尿，失禁の増加や患者自身による蓄尿もれなどにより，正確な24時間蓄尿が困難なことがある．1日尿量が少ないとき，Ccr 実測値と推定値のばらつきが大きいとの報告もある．今回は尿道留置カテーテルを使用している患者や蓄尿が可能と判断された患者の症例を対象とし，努めて正確な採尿を試みた．しかしながら，本来行うべきクリアランス法の実施には正確な蓄尿と安静を要し，判定に時間がかかるため実際の外来診療では実施困難なことが多い．従って Scr より Ccr を推定する種々の方法が提案されてきた．今回検討した安田の式，Cockcroft and Gault の式，折田の式，Walser の式は代表的な推定式であり Scr 値，性別，年齢，体重より Ccr を推定できる．C&G 式は欧米で最も広く用いられており欧米人によい相関を示して

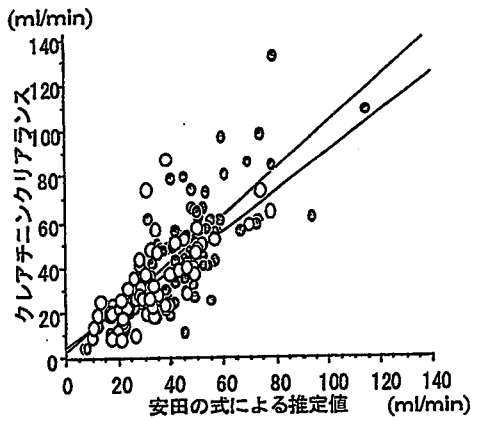

図1　安田の式　84歳以下と85歳以上の比較
○85歳以上；Y = 4.57 + 0.860X（r = 0.718）
●84歳以下；Y = 1.85 + 1.007X（r = 0.761）

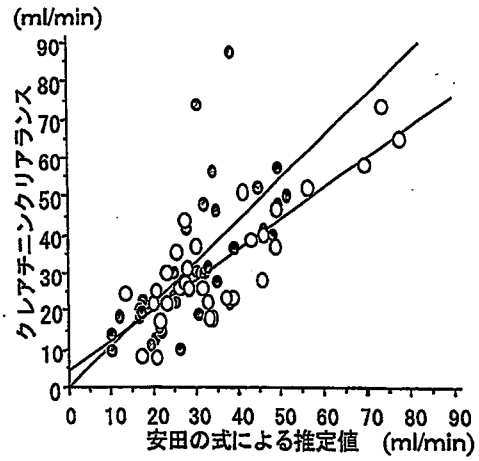

図3　安田の式　85歳以上の性差
○男性；回帰式 Y = 4.09 + 0.796X（r = 0.840）
●女性；回帰式 Y = 0.21 + 1.088X（r = 0.678）

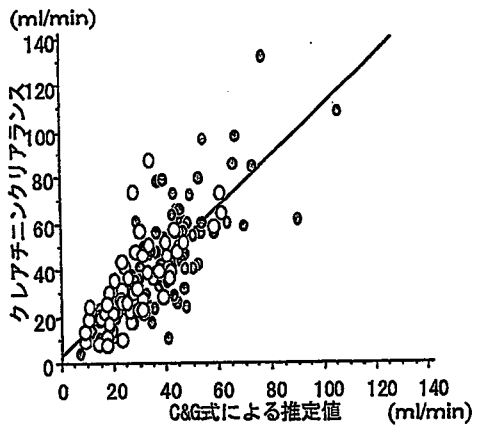

図2　C&G式　84歳以下と85歳以上の比較
○85歳以上；Y = 3.20 + 1.078X（r = 0.739）
●84歳以下；Y = 3.33 + 1.082X（r = 0.761）

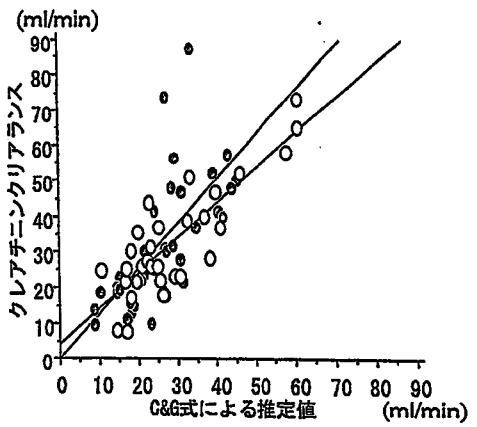

図4　C&G式　85歳以上の性差
○男性；回帰式 Y = 4.07 + 0.988X（r = 0.841）
●女性；回帰式 Y = − 0.09 + 1.262X（r = 0.690）

いる．今回の検討でも超高齢者における相関が0.739と最もよい相関を示した．この原因として日本人の体格が欧米化してきたことやC&G式作成時の対象年齢が18～92歳と超高齢者も含まれていること，作成時の対象症例数が多いことが考えられる．C&Gの式に対して他の3式はいずれもその後に発表されたもので，安田の式は1.4mg/dl以下の血清クレアチニン値を示す高齢者に限定して式を求めたもので，腎不全患者は含めずに高齢者の腎機能を推定しようとしたものである[2]．一方，Walserの式は血清クレアチニン値を2.0mg/dl以上におき，腎不全患者のみを対象としている[5]．堀尾らの式は腎疾患患者を対象として，推定式にBMIの項を加えて肥満の特徴加味して作成された[4]．したがって，今回の対象のように腎機能が広範囲に亘る場合，C-Gの式以外では，いずれもずれが出てしまう結果となったのは，式の作成経緯による要素も大きいと考えられる．

今回，臨床の現場では安定した時期より外来や急性期での腎機能評価を必要とするため，疾患による除外は設けず，脳血管障害，感染症，経口摂取不良，利尿剤，補液などの様々な基礎疾患，治療を有する高齢者を対象に行った．推定式と実測値の乖離に関して，実測値が大きい場合は，輸液や降圧剤など腎血流量を増加させる治療が関与していた場合が多かった．この場合は臨床的には大きな実害は考えられない．一方，実測値が推定式より小さい場合は，相対的な薬物の過量投与など安全管理上

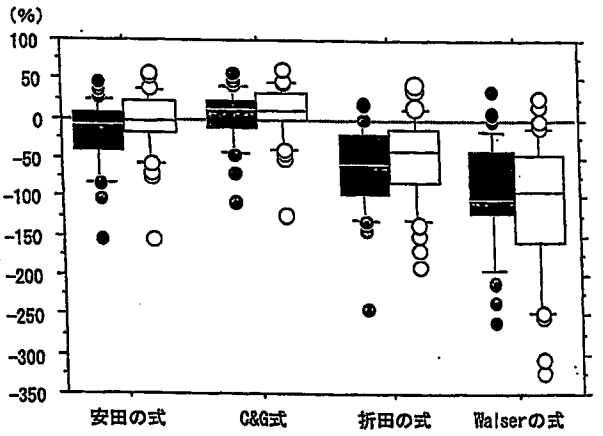

図5　超高齢者男女別において各推定式による推定値と実測値とのずれを箱ひげ図で％表示したもの
縦軸（実測値－推定値）×100/実測値
●男性
○女性

も問題となる．今回の検討では，腎不全，癌，乏尿，コントロール不良の糖尿病，胸水，腹水など複数の病態が重なる重症例で，有効循環血液量も日々変動しうる症例であった．このような症例に救急外来で遭遇した場合，血清クレアチニンから推定されるCcrの精度が低い可能性があることを銘記すべきであろう．Scrについては6.9までの高値も含まれているが，高値を除いた検討を行っても相関に大きな変化は見られなかった．全式において84歳までの前期及び後期高齢者群と85歳以上の超高齢者群に分け，相関を比較したところ，超高齢者群での相関が低い傾向にあり，超高齢者群での合併疾患の増加の影響が示唆される．これらを考慮しても，4種の推定式を比べると相関係数が最も高いC&G式が本邦超高齢者におけるCcr推定式として最適と考えられた．

超高齢者群を男女にわけC&Gの相関係数を比較したところ，男性0.841女性0.690と男性の相関が高い傾向にあった．また，回帰係数を比較したところ男性ではC&G式，女性では安田の式が1に近い値を示した．85歳以上の男性に安田の式を用いると過大評価する可能性があり，85歳以上の女性にC&G式を用いると過小評価する可能性がある．

一方，前期及び後期高齢者群の回帰係数を比較したところ男女ともに安田の式が1に近い値を示した．超高齢者の筋肉量について本邦での正確なデータは少ないが，中島らによれば70歳以降男性では上腕筋周囲，上腕筋面積が急速に減少するが女性ではほとんど変わらない[7]ことから女性の筋肉減少が時代とともに変化し，推定式の再構築が迫られている可能性があり，今後の検討課題と思われた．

本研究の限界として，膀胱留置カテーテルの適応がない蓄尿不可能症例を除外していることがあげられる．具体的には尿失禁症例や，認知症などが含まれるが，これらの症例に対してカテーテル留置を行ってクレアチニンクリアランスを測定し，高齢者全体に対するの推定式の良否を判断する研究は今後の課題であろう．

結　語

超高齢者において，正常値から腎不全を含む範囲の腎機能の判定に，24時間クレアチニンクリアランスの実測値と，すでに発表されている4つの式から求めた推定値とを比較して，超高齢者での推定式の有用性を検討した．4つの推定式のうち，C-Gの式はこの研究の目的にもっとも合致していた．一方，安田の式（高齢者，Scr: 1.4mg/dl以下），Wの式（Scr 2.0mg/dl以上）はいずれもその適用の目的の範囲で，また堀尾の式は腎疾患群内で有用と思われた．

全体として，臨床的に使用するうえでC&G式が最も優れているが，超高齢者への適用に当たっては，10%程度，推定値が低く求まるので，補正が望ましい．

今後超高齢者については，体格，サルコペニアの時代的変遷を考慮して改訂していく必要がある．

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Creatinine clearance estimation in the extremely elderly subjects

Shunichi Hirayama[1], Reiko Kikuchi[2], Shinichiro Inoue[2], Daisuke Tsukahara[2], Yumi Suemitsu[2], Yoshio Kobayashi[2], Yoichi Sugiyama[2], Hiroshi Hasegawa[2], Koichi Kouzaki[2], Gosuke Inoue[3] and Kenji Toba[2]

Abstract

Background: It has been reported that elderly outpatients take at least 6 different kinds of medication.

Purpose: To know which formula will best predict creatinine clearance, because 24-hour urine collection is difficult for elderly outpatients.

Patients and Methods: We compared four types of formulae (Cockcroft & Gault, Yasuda, Orita, Walser) to estimate creatinine clearance using serum creatinine of 143 elderly inpatients (73 men, 70 women, mean age 82.9±8.6 years old) including 67 extremely elderly people with various underlying diseases.

Result: The formula of Cockcroft and Gault showed the best correlation with creatinine clearance in the extremely elderly subjects ($r=0.74$) as well as in people under 85 years ($r=0.76$). However, the estimated values of the extremely elderly women were lower than actual creatinine clearance.

Conclusion: The formula of Cockcroft and Gault is the best predictive equation of creatinine clearance, except in the extremely elderly women.

Key words: *Extremely elderly, Creatinine clearance, Predicting formula, Cockcroft & Gault's formula, Yasuda's formula*
(Nippon Ronen Igakkai Zasshi 2007; 44: 90–94)

1) Tokyo University of Pharmacy and Life Science
2) Department of Geriatric Medicine, Kyorin University, School of Medicine
3) Department of Internal Medicine, Higashimurayama Nursing Home

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Serum Levels of S-Glutathionylated Proteins as a Risk-Marker for Arteriosclerosis Obliterans

Kazuki Nonaka, MD; Noriaki Kume, MD††; Yoshishige Urata, PhD; Shinji Seto, MD*; Takaaki Kohno, BS*; Sumihisa Honda, PhD*; Soji Ikeda, MD*; Takahiro Muroya, MD**; Yosihiko Ikeda, MD†; Yoshito Ihara, MD; Toru Kita, MD††; Takahito Kondo, MD

Background Oxidative stress plays a role in the development of chronic peripheral arterial disease (PAD) because under these conditions redox regulation is impaired, inducing the S-glutathionylation of proteins. A method of estimating the levels of S-glutathionylated proteins has been developed using biotinylated glutathione S-transferase, which allows the study of their crucial role in the oxidative stress-related progression of PAD.

Methods and Results The serum levels of S-glutathionylated proteins were examined in 41 patients with arteriosclerosis obliterans (ASO) and 38 age-matched non-ASO patients using biotinylated glutathione S-transferase. The levels were higher in the patients with ASO, even early on, and positively correlated with the ankle/brachial index. In vitro, the levels of S-glutathionylated proteins were reduced in the presence of glutathione and glutaredoxin.

Conclusions Serum levels of S-glutathionylated proteins are a sensitive risk-marker for ASO at an early stage. (*Circ J* 2007; **71**: 100–105)

Key Words: Arteriosclerosis obliterans; Oxidative stress; S-glutathionylation

The number of patients suffering from arteriosclerosis obliterans (ASO) is anticipated to increase, accompanying the increase in incidence of risk factors such as obesity, hypercholesteremia, diabetes, and hypertension[1] Pathologically, ASO derives from atherosclerosis, and complete occlusions by fresh or old thrombi are often observed. Treatment includes anticoagulants, antiplatelet drugs, and vasodilators, and in the advanced stages percutaneous transluminal angioplasty, bypass surgery, and prosthetic arterial grafts have been used. New approaches include intravenous administration of prostaglandin E_1[2] or gene therapy with hepatocyte growth factor[3] both aimed at increasing peripheral blood flow. Most patients with ASO have no apparent clinical symptoms early on, but diagnosis at the early stage is essential for preventing progression. Unfortunately, there are currently no specific and sensitive markers for ASO, so the aim of this study was to find a new risk-marker for the diagnosis of ASO in the earlier stages.

The development of atherosclerosis is induced by severe damage to endothelial cells from various pro-inflammatory cytokines, adhesion molecules, or sheer stress, for example[4–6] Furthermore, oxidative stress is believed to play a crucial role in the progression of peripheral arterial disease (PAD)[7] because it induces modifications of cellular components such as proteins, lipids, and DNA, leading to cell dysfunction or apoptosis. Most of the risk factors for PAD, such as smoking, obesity, hypertension, diabetes, and hypercholesterolemia, create oxidants that damage endothelial cells[7] The cysteine thiols of proteins are easily modified by oxidative stress when the antioxidative systems are suppressed and under oxidative stress caused by reactive oxygen species or nitrogen oxide species, it is the sulfhydryl residues of proteins that are most susceptible. In response, the sulfhydryl groups are oxidized to form disulfides in a reaction with the reduced form of glutathione disulfide (GSSG) or converted irreversibly to sulfenic, sulfinic, and sulfonic acid derivatives[1] S-Glutathionylated proteins reported to date include glyceraldehyde-3-phosphate dehydrogenase[2] annexin A2[3] protein kinase C[4] and carbonic anhydrase III[5] The S-glutathionylation of proteins is initiated in the presence of GSSG[8] The S-glutathionylation of the sulfhydryl groups changes a protein's function, and the process is regulated by thioredoxin (TRX) or glutathione (GSH)/glutaredoxin (GRX). Such modifications of protein-thiols by oxidative stress are speculated to occur in patients with PAD; however, no data on changes in the levels of serum S-glutathionylated proteins have been reported for patients with peripheral or cardiovascular diseases such as stroke, coronary artery disease, and end-stage renal disease. We are interested in the S-glutathionylation of proteins in ASO patients and so the aim of the present study was to evaluate the serum levels in these patients, as a risk-marker for ASO in the early stages, because elevation supposedly reflects redox imbalance.

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*Department of Biochemistry and Molecular Biology in Disease, *Department of Radiation Epidemiology, Atomic Bomb Disease Institute, **Department of Internal Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, †Tagawa Municipal Hospital, Tagawa and ††Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan*

Mailing address: Takahito Kondo, MD, Department of Biochemistry and Molecular Biology in Disease, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. E-mail: kondo@net.nagasaki-u.ac.jp

Table 1 Characteristics of Patients With ASO

	Non-ASO patients	ASO patients (Fontaine)		
		Stage 1	Stage 2	Stage 3
Patients, n	38	9	22	10
ABI (mean±SD)	0.94±0.02	0.72±0.07	0.41±0.13	0.21±0.06
Age (mean±SD), years	66±11	73±8	72±7	71±5
Male sex, n (%)	15 (39)	6 (67)	16 (73)	8 (80)
Risk factors, n (%)				
Hypertension	17 (45)	7 (78)	19 (86)†	9 (90)†
Diabetes	23 (61)	3 (33)	6 (27)‡	3 (30)
Smoking	9 (24)	6 (67)§	15 (68)§	5 (50)
Hypercholesterolemia	16 (42)	4 (44)	9 (41)	5 (50)
Chronic renal failure on hemodialysis	0	0	0	0
Angina	4 (11)	2 (22)	4 (18)	3 (30)
Lipid profile (mean±SD)				
Total cholesterol, mg/dl	253±45	198±23*	205±28*	202±17*
LDL-C, mg/dl	153±46	111±24¶	119±23¶	116±14¶
hs-CRP (mean±SD), ng/ml	2.19±0.42	3.17±1.01	3.57±0.85#	4.30±0.99#

Values for hs-CRP were transformed in logarithm of 10. One-way ANOVA was followed up with Tukey-Kramer pairwise comparisons among means.

*†p<0.05 for comparison with non-ASO patients; ‡p<0.05 for comparison with non-ASO patients; §p<0.05 for comparison with non-ASO patients; *p<0.005 for comparison with non-ASO patients; ¶p<0.05 for comparison with non-ASO patients; #p<0.05 for comparison with non-ASO patients.*

ASO, arteriosclerosis obliterans; ABI, ankle/brachial index; LDL-C, low-density lipoprotein-cholesterol; hs-CRP, high-sensitivity C-reactive protein.

Methods

Patient Sample

We enrolled 41 patients diagnosed with ASO. All of them had the characteristic complaints of chronic limb ischemia, including intermittent claudication, rest pain, or non-healing ischemic ulcers (Fontaine I, n=9; Fontaine II, n=22; Fontaine III, n=10) as confirmed by angiography. Of the patients visiting hospital without apparent PAD, we recruited 38 age-matched controls. All participants gave written informed consent and prior to the commencement of the present study, the protocol was approved by the ethics committees of all the participating universities and hospitals.

Measurement of Ankle/Brachial Index (ABI)

Blood pressure, heart rate, and ABI were measured using the Form pulse wave velocity (PWV)/ABI non-invasive vascular screening device (Nihon Colin Inc, Tokyo, Japan) after the subject had rested supine for at least 20 min. ABI was calculated 2 or 3 times for both legs and averaged; an ABI <0.9 was considered to indicate the presence of disease.

Immunoblot Analysis

Unless otherwise indicated, 20 μg samples of serum was used. Protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Samples were electrophoresed on 5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) in the absence of dithiothreitol (DTT) and the proteins in the gels were transferred onto nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (10 mmol/L Tris-HCl [pH7.5] and 0.15 mol/L NaCl; TBS) containing 0.05% Tween 20, v/v (TBST), and 5% (w/v) nonfat dry milk, then reacted with primary antibodies in TBST containing 3% (w/v) bovine serum albumin overnight with constant agitation at 4°C. After several washes with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-IgG antibodies. Proteins in the membranes were then visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions. Levels of high-sensitivity C-reactive protein (hs-CRP) were determined in the same serum samples used for sLOX-1, with a commercially available electrochemiluminescent immunoassay kit (F. Hoffman-La Roche Ltd).

Detection of S-Glutathionylated Proteins by Biotin-Glutathione S-Transferase (GST) on Blotted Membranes

Serum levels of S-glutathionylated proteins were estimated according to the methods described by Cheng et al[8] using biotinylated GST. Serum samples were collected serially and stored at −80°C until assays were performed. Of each sample, 20 μg/lane were subjected to 5% SDS-PAGE under non-reducing conditions. The proteins in the gels were transferred onto nitrocellulose membranes, which were blocked in phosphate bufferd salin (PBS) containing 0.1% Tween 20, v/v, and 5% nonfat dry milk, then treated with BSA containing 5% (w/v) bovine serum albumin for 2 h at room temperature and further incubated with 30 mg/ml biotin-GST overnight. After several washes with PBS, the membranes were incubated with HRP-conjugated streptavidin (1:1,000 dilution) for 1 h at room temperature. Peroxidase activity was detected after treatment with 2 mmol/L hydrogen peroxide and 0.6 mg/ml 4-chloro-1-naphthol in PBS.

Statistical Analysis

Statistical analysis was performed using Stat-View (version 4.5, Abacus Concepts Inc, Calabasus, CA, USA) and R. The 1-way ANOVA was used to compare continuous variables, with the Tukey-Kramer test for multiple comparisons, and 2-way cross-tabulation with the chi-square test was used for binary variables, when appropriate, to compare differences between groups. Statistically significant differences among groups were analyzed by the Kruskal-Wallis test with Dunn's test. When S-glutathionylated proteins

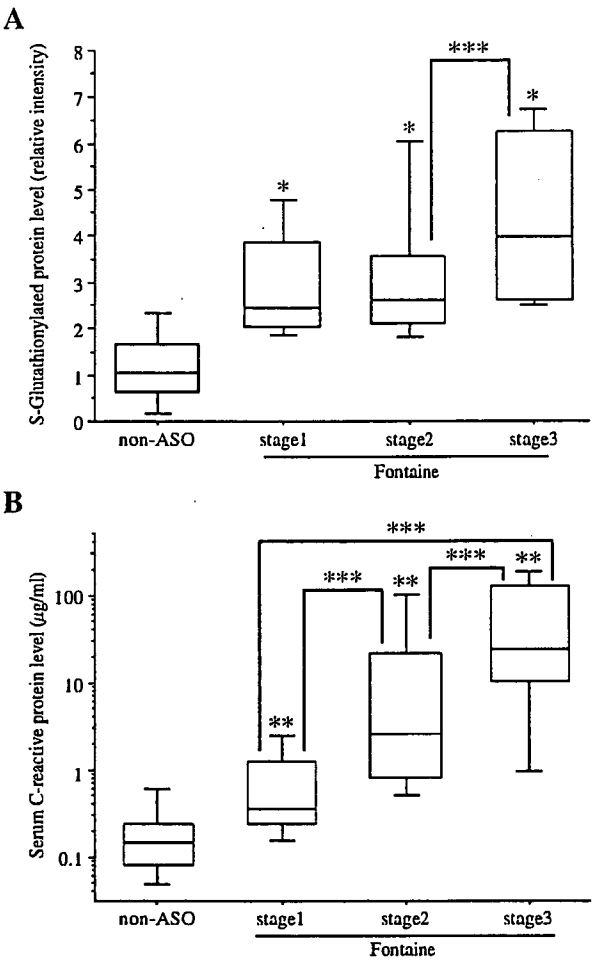

Fig 1. Serum levels of S-glutathionylated proteins and high-sensitivity C-reactive protein (hs-CRP). S-glutathionylated protein levels are expressed as relative intensity compared with non-ASO patients (A), and hs-CRP (μg/ml) levels (B) were determined in 41 ASO patients (9 at Fontaine stage 1, 22 at stage 2, and 10 at stage 3) and 38 non-ASO patients. Data are indicated in box plots. Center horizontal lines indicate median values; upper and lower edges of outer boxes, 25th and 75th percentiles; and lower and upper bars, 10th and 90th percentiles. Statistically significant differences among the 4 groups by Kruskal-Wallis test with Dunn's test. *p<0.0001 vs non-ASO patients; **p<0.05 vs non-ASO patients; ***p<0.05. ASO, arteriosclerosis obliterans.

were undetectable by immunoblot analysis, a score of 0 was assigned. Any association between S-glutathionylated proteins and hs-CRP, total cholesterol or low-density lipoprotein (LDL)-cholesterol (C) was evaluated with Spearman's rank correlation coefficient. Logarithmic values of hs-CRP were used as variables for statistical analyses. The effect of S-glutathionylated proteins on ABI was analyzed by using a multiple linear regression model with hyperlipidemia, hypertension, smoking, diabetes mellitus, and hs-CRP as covariates. The squared multiple correlation coefficient (R^2) was calculated as a goodness-of-fit measure. Values of $p<0.05$ were considered statistically significant.

Results

Clinical Characteristics of the Study Group

Table 1 summarizes age, gender, conventional vascular risk factors, ABI, lipid profile, and levels of hs-CRP. Patient characteristics, including age and the incidence of hypercholesterolemia and angina, were comparable between the ASO and non-ASO groups. The ratio of males to females was higher in the ASO groups. Of the risk factors, the rate of hypertension was higher at Fontaine stages 2 and 3 in the ASO groups than in the non-ASO group ($p<0.05$), and the rate of smoking was higher at Fontaine stages 1 and 2 in the ASO groups ($p<0.05$). There was no difference in the rate of angina between the ASO and non-ASO groups. In this study, patients with chronic renal failure on hemodialysis were excluded. The serum concentrations of total cholesterol and LDL-C were lower in the ASO groups than in the non-ASO group ($p<0.05$).

Serum Levels of S-Glutathionylated Protein

Fig 1A shows the estimated S-glutathionylated protein levels in serum samples from the ASO groups. Statistically significant differences were found among the 4 groups (Kruskal-Wallis test). The median level of S-glutathionylated proteins was 1.06 in non-ASO patients, 2.46 at stage 1, 2.62 at stage 2, and 3.97 at stage 3. The number of males in the non-ASO group was less than in the ASO groups; however, in a preliminary study, there was no difference in the levels of S-glutathionylated proteins between the sexes (data not shown). The levels were increased at every stage of ASO compared with the non-ASO patients ($p<0.0001$). A significant difference in the levels of S-glutathionylated proteins was observed between stages 2 and 3 ($p<0.05$). Table 1 and Fig 1B show the serum levels of hs-CRP in the ASO groups; they were higher than in the non-ASO patients ($p<0.0001$) and increased as the disease developed ($p<0.05$).

Fig 2 shows a typical result of the analysis of S-glutathionylated proteins using biotin-GST. SDS-PAGE profiles did not differ between sera from non-ASO patients and sera from ASO patients under reduced (Fig 2A, lanes 2, 3) or non-reduced (lanes 4, 5) conditions. S-glutathionylated protein bands were detected more in ASO patients than in non-ASO patients under non-reduced conditions (Fig 2B). In vitro, levels of S-glutathionylated proteins were reduced weakly in the presence of the GSH/GSSG system (Fig 2C, lane 2), and strongly in the presence of the GSH/GSSG system and GRX (lane 3). This suggests that the increase in the serum levels of S-glutathionylated proteins reflects a reduced redox regulation in ASO patients. Immunoprecipitation of proteins by anti-apolipoprotein B100 (apoB100) and treatment with biotin-GST revealed that apoB100 is S-glutathionylated in ASO (Fig 2D), which suggests that the S-glutathionylation of proteins in serum involves apoB100.

The serum levels of total cholesterol and LDL-C were higher in the non-ASO patients than in the ASO patients; however, there was no correlation between the levels of S-glutathionylated proteins and those of total cholesterol or LDL-C. Similarly, the levels of S-glutathionylated proteins did not relate to the levels of triglyceride in serum (data not shown).

Relationship Between S-Glutathionylated Proteins and ABI

The relationship between S-glutathionylated proteins and ABI was analyzed using a multiple linear regression model with covariates (Table 2). The coefficient, standard error, and p-value of S-glutathionylated proteins were −0.0455, 0.0173, and 0.0105, respectively. Similarly, the p-value of both hypertension and smoking was less than 0.05. The data suggest that formation of S-glutathionylated

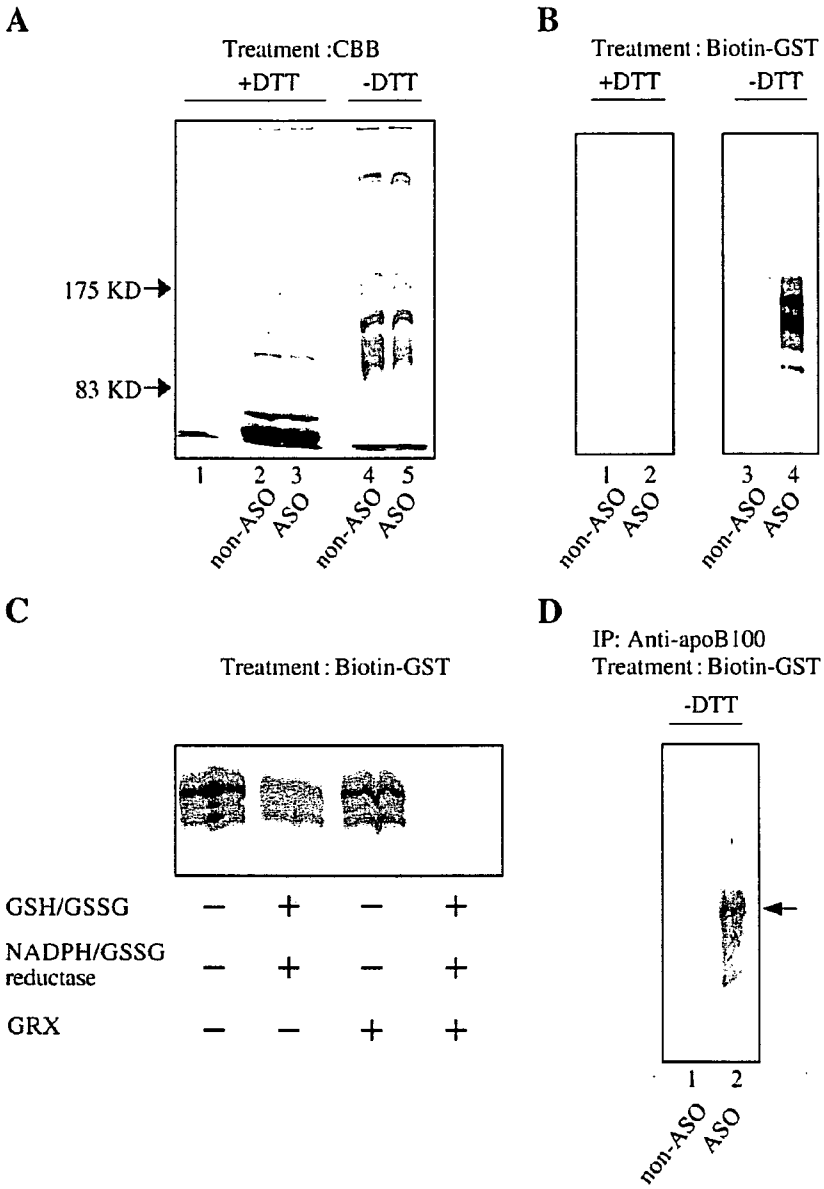

Fig 2. Characteristics of S-glutathionylated proteins. Serum samples from a patient with ASO and another with non-ASO was subjected to 5% SDS-PAGE and analyzed using biotin-GST. (A) CBB stain. Lane 1, molecular weight standard; lane 2, non-ASO with DTT; lane 3, patient with DTT; lane 4, non-ASO without DTT; lane 5, patient without DTT. (B) Staining using biotin-GST under reducing conditions (1 and 2) and non-reducing conditions (3 and 4). (C) Effect of the GSH/GRX system on the S-glutathionylated proteins. From the serum sample of a patient with ASO 1 μl was treated in buffer containing components of the GSH/GRX system for 30 min at 30°C. GSH/GSSG, 1 mmol/L GSH and 0.05 mmol/L GSSG; NADPH/GSSG reductase, 1 mmol/L NADPH and 1.2 units of GSSG reductase; 1 μg of GRX (16 samples were subjected to 5% SDS-PAGE under non-reducing conditions and analyzed using biotin-GST. (D) Serum was immunoprecipitated by anti-apoB100 antibody, subjected to 5% SDS-PAGE under non-reducing conditions, and analyzed using biotin-GST. Lane 1, non-ASO patient; lane 2, ASO patient. The arrow indicates a band corresponding to apoB100 protein. ApoB100, apolipoprotein B100; ASO, arteriosclerosis obliterans; CBB, coomassie brilliant-blue; DTT, dithiothreitol; GRX, glutaredoxin; GSH, glutathione; GST, glutathione S-transferase; GSSG, the reduced form of glutathione; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels.

Table 2 Multiple Linear Regression Model for ABI

Variables	*Coefficient*	*Standard error*	*p value*
S-glutathionylated proteins (relative intensity)	*−0.0455*	*0.0173*	*0.0105*
*Hypertension**	*−0.189*	*0.0622*	*0.00338*
*Diabetes**	*0.0275*	*0.0503*	*0.587*
*Hypercholesterolemia**	*−0.0229*	*0.0514*	*0.657*
*Smoking**	*−0.170*	*0.0512*	*0.00142*
*hs-CRP (ng/ml)***	*−0.0513*	*0.0286*	*0.0774*

**Positives are defined as 1. Negatives are defined as 0. **Values for hs-CRP were transformed in logarithm of 10. Abbreviations see in Table 1.*

proteins in serum is involved in the progress of ASO.

Discussion

As to the diagnosis of ASO, several tests, such as angiography, estimations of ABI and PWV, and measurements of circulating levels of hs-CRP, have been used to detect PAD. However, these estimations are not sufficient to predict the development of ASO in its earlier stages.

Oxidative stress is a principle cause of aging and the development of diseases such as inflammation, infection, cancer, and cardiovascular disorders[9,10] Exogenous or endogenous sources of oxidative stress and weakened antioxidative defenses can damage macromolecules such as

DNA, lipids, and proteins. The levels of molecules modified by oxidative stress can be estimated; however, there are currently no sensitive and specific methods to evaluate the oxidative stress-induced development of cardiovascular diseases.

The redox system regulates certain protein functions and protects cells from H_2O_2-induced apoptosis[16] TRX is a protein that is ubiquitously expressed in all living cells and which fulfils a variety of biological functions related to cell proliferation and apoptosis[17] Increases in serum TRX levels have been found in patients with various coronary risk factors, such as smoking, hypertension, and hypercholesterolemia[18] Increases in S-glutathionylated proteins have been found in ischemic preconditioned hearts[19] Those reports suggest that chronic oxidative stress may be involved in the progression of the coronary diseases associated with risk factors. As to the role of GSH/GRX, we previously found that the anti-apoptotic activity of Akt is regulated by the GSH/GRX system inside the cell[16] which led us to speculate that an imbalance of the redox state in serum reflects an impairment of circulatory compartments by oxidative stress, and we became interested in estimating the levels of S-glutathionylated proteins in serum as a marker for the risk of developing peripheral vascular damage. In the present study, serum levels of S-glutathionylated proteins were elevated in the earlier stages of ASO (Fig 1A). Levels of S-glutathionylated proteins in sera from ASO patients were reduced in the presence of the GSH/GRX system (Fig 2C). These results strongly suggest that during the development of ASO, chronic oxidative stress induces an imbalance of the redox state and protein thiols are oxidized in the serum of patients with ASO, although the mechanism of redox regulation to maintain the reduced form of cysteine thiols is not well understood. The application of anti-oxidant therapies, such as α-tocopherol[18] statins[20] or exercise[21] may improve the redox imbalance and reduce the levels of S-glutathionylated proteins in ASO. If so, estimation of S-glutathionylated proteins is useful as a marker for the success of therapies and trials may be warranted.

Redox-active cysteine residues in the albumin of human serum have been reported;[22] however, under the experimental conditions used in the present study, we could not identify S-glutathionylated albumin (data not shown). We found that apoB100 protein is S-glutathionylated (Fig 2D). At present, it is unclear if the thiol-modification of apoB100 affects its function.

The method we used for the estimation of S-glutathionylated proteins used biotinylated GST. However, methods using electrophoresis are neither simple nor sensitive. Attempts have been made to detect S-glutathionylated proteins by a proteomic approach using ^{35}S-labeled GSH in vitro, but this is not a convenient method[23] Further development of a widely applicable method, such as enzyme-linked immunosorbent assay, is required for use with clinical samples.

ASO is an atherosclerotic peripheral occlusive disease. Oxidized LDL (ox-LDL) appears to play a key role in atherogenesis[24] A circular Ox-LDL, a product of oxidative stress, has been reported in patients with hyperlipidemia[25,26] In the present study, there was no relation between lipid metabolism and levels of S-glutathionylated proteins (data not shown), and a pathological comparison of S-glutathionylated proteins with ox-LDL was not conducted.

The relationship between various risk-markers and the development of ASO was analyzed with a multiple regression model (Table 2). The data suggested that levels of S-glutathionylated proteins in serum are a risk-marker for ASO. Similarly, cigarette smoking was found to correlate with a decrease in ABI, which is consistent with a report that smoking induces low-grade inflammation and thrombogenicity[27] as well as chronic obstructive pulmonary disease[28] It should be taken into account that there are many other factors influencing the progress of ASO, such as drugs, duration of accompanying diseases, genetic background, etc and the analysis here is not sufficient to rule out other risk factors; however, the increase in the serum levels of S-glutathionylated proteins may, at least in part, reflect progression of ASO.

In summary, the S-glutathionylation of proteins in serum may reflect the redox imbalance induced by oxidative stress and play a role in the development of ASO.

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Alpha-defensin enhances expression of HSP47 and collagen-1 in human lung fibroblasts

Sumako Yoshioka [a], Hiroshi Mukae [a,*], Hiroshi Ishii [a], Tomoyuki Kakugawa [a], Hiroshi Ishimoto [a], Noriho Sakamoto [a], Takeshi Fujii [a], Yoshie Urata [b], Takahito Kondo [b], Hiroshi Kubota [c], Kazuhiro Nagata [c], Shigeru Kohno [a]

[a] *Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki, 852-8501 Japan*
[b] *Department of Biochemistry and Molecular Biology in Disease, Nagasaki University School of Medicine, Nagasaki, Japan*
[c] *Department of Molecular and Cellular Biology, Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan*

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Abstract

Neutrophils and lung fibroblasts are thought to play a role in the pathogenesis of pulmonary fibrosis. We reported previously that heat shock protein 47 (HSP47), a collagen-specific molecular chaperon, and collagen-1 synthesis were involved in pulmonary fibrosis, and that plasma levels of α-defensins (HNP; human neutrophil peptide), cationic proteins with antimicrobial and cytotoxic activity in neutrophils, were significantly higher in patients with idiopathic pulmonary fibrosis than in control subjects. Here, we investigated the direct effect of HNP-1 *in vitro* on the expression of HSP47 and collagen-1 in human lung fibroblasts (NHLF). HNP-1 at 5 μg/ml induced fibroblast proliferation but at concentrations >50 μg/ml, HNP-1 reduced cell viability. Incubation of NHLF with 10 to 25 μg/ml of HNP-1 for 24-h increased the expression of HSP47 and collagen-1 mRNAs ($p<0.05$). The levels of HSP47 protein also increased significantly at 50 μg/ml, and those of collagen-1 protein increased at 10 to 50 μg/ml of HNP-1 ($p<0.05$). The mitogen-activated protein kinase (MAPK) signaling pathway in NHLF was activated by HNP-1 stimulation, but inhibitor of MEK (PD98059) did not block HNP-1-induced HSP47 protein production. Our results suggest that α-defensin is a fibrogenic mediator that promotes collagen synthesis through the upregulation of HSP47 and collagen-1 in lung fibroblasts and participates in the pathogenesis of neutrophil-induced pulmonary fibrosis.

Keywords: Human neutrophil peptide-1; HSP47; Collagen synthesis; Pulmonary fibrosis

Introduction

Neutrophils are considered to play a pathologic role in acute and chronic injurious diseases of the lung. A marginal increase of neutrophils in the respiratory tract can be associated with major damage and irreversible architectural changes in the lung (Sibille and Marchandise, 1993). Idiopathic pulmonary fibrosis (IPF) is a progressive and usually fatal lung disease characterized by patchy fibrotic areas with fibroblast proliferation and extracellular matrix remodeling, which result in irreversible distortion of the lung architecture. Increasing scientific evidence defines the importance of neutrophils in the pathogenesis of pulmonary fibrosis (Crystal et al., 1984; Hunninghake et al., 1981; Obayashi et al., 1997; Turner-Warwick and Haslam, 1987; Wells et al., 1998). For example, large numbers of neutrophils were found in the bronchoalveolar lavage (BAL) fluid (Crystal et al., 1984; Hunninghake et al., 1981) and lung tissue of patients with IPF (Hunninghake et al., 1981; Obayashi et al., 1997). Furthermore, BAL neutrophil counts in IPF patients correlated with the severity of the disease as determined by chest CT (Wells et al., 1998), poor treatment outcome (Turner-Warwick and Haslam, 1987) and disease activity (Crystal et al., 1984). However, other studies reported that the number and proportion of neutrophils in BAL fluid did not correlate with the activity of neutrophil alveolitis and that they had limited prognostic value (Boomars et al., 1995; Schwartz et al., 1994). Thus, the exact role of neutrophils in the pathogenesis of IPF remains unclear.

* Corresponding author. Tel.: +81 95 849 7273.
E-mail address: hmukae@net.nagasaki-u.ac.jp (H. Mukae).

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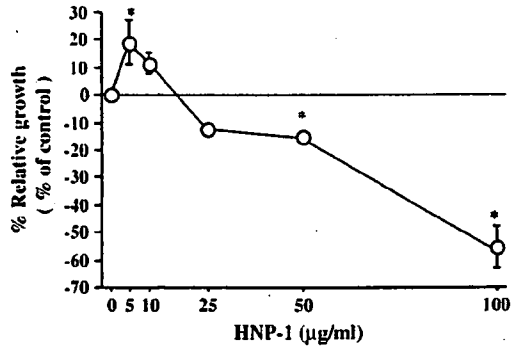

Fig. 1. Influence of HNP-1 on proliferation of normal human lung fibroblasts (NHLF) measured by Alamar Blue reduction assay. NHLF were separately incubated for 24 h with 0 (control) to 100 μg/ml of HNP-1. The growth levels were compared with non-treated control cells and the resulting ratio is shown as the percentage change from control values. Data are mean±SEM of four experiments. *$p<0.05$, compared with the control.

NP-40, 10% glycerol, 0.2 mM PMSF, 1 μg/ml pepstatin and leupeptin). The lysates were centrifuged at 15,000 ×g for 10 min at 4 °C and the supernatant was used as the cytoplasmic extract. Each extract was subjected to SDS-PAGE using 10% polyacrylamide gels. After electrophoresis the fractionated protein was transferred to a nitrocellulose transfer membrane (Pall Corporation, East Hills, NY). The membrane was blocked for 60 min at room temperature in 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20, and then incubated overnight at 4 °C in 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 with 1 μg/ml the mouse monoclonal anti-HSP47 antibody (StressGen Biotechnologies, Victoria, BC, Canada), 1 μg/ml mouse monoclonal anti-ERK1/2 antibody (Cell Signaling, Beverly, MA) or 1 μg/ml mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Excess antibody was washed away with Tris-buffered saline (pH 7.5) containing 0.01% Tween 20. The nitrocellulose membranes were incubated for 30 min at room temperature in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20 and the secondary antibody (1:2000 dilution in Tris-buffered saline, pH 7.5, containing 0.05% Tween 20) (Peroxidase-conjugated rabbit anti-mouse immunoglobulins) (DAKO, Kyoto, Japan) followed by washing in Tris-buffered saline (pH 7.5) containing 0.01% Tween 20. The detection was performed using the ECL Plus Western Blotting Detection Reagents kit (Amersham Biosciences Corp). The intensity of HSP47 was correlated using mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA) calculated by densitometry.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected 24 h after addition of HNP-1 or TGF-β1 suspension, centrifuged and stored at −80 °C. Collagen-1 C-terminal telopeptide levels were measured using

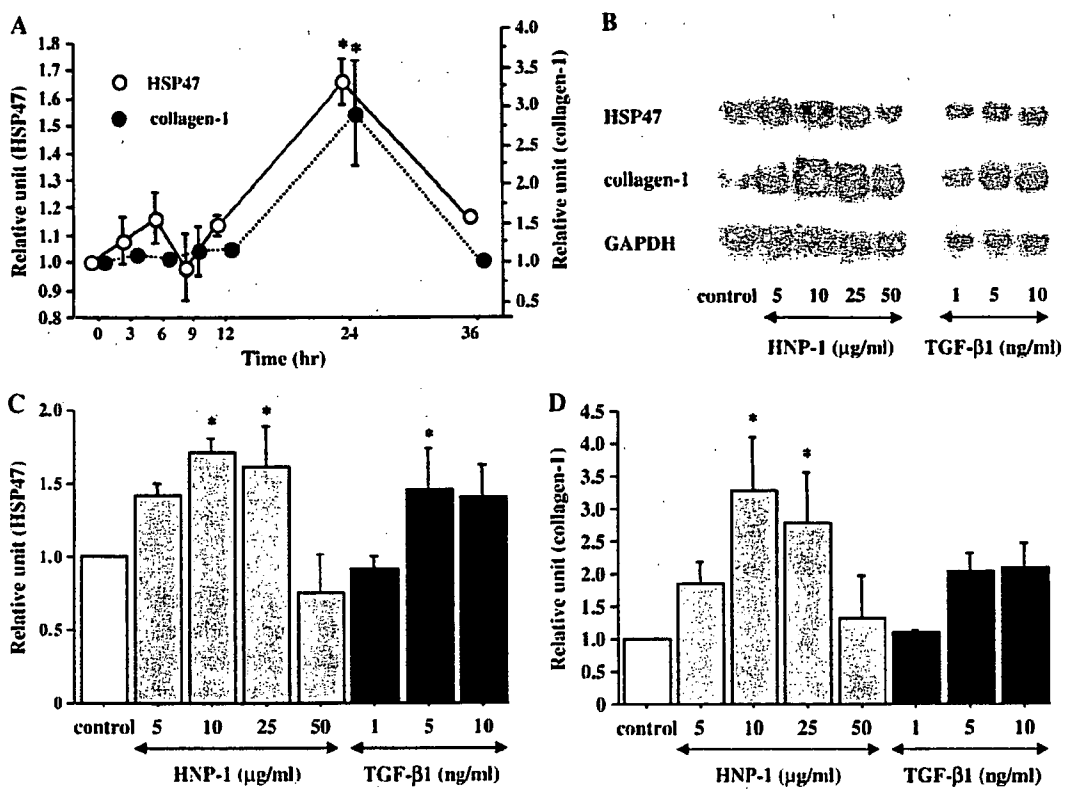

Fig. 2. Time course analysis of HSP47 and collagen-1 mRNA expression in NHLF treated with 10 μg/ml of HNP-1 by northern blotting (A). Representative autoradiographs of northern blot (B) showing mRNA expression of HSP47 and collagen-1 in NHLF after 24 h incubation with medium alone (control), 5, 10, 25 and 50 μg/ml of HNP-1 and 1, 5 and 10 ng/ml of TGF-β1. Results of densitometric analysis of bands on autoradiographs are shown in Fig. 2C (HSP47) and Fig. 2D (collagen-1). The density of the bands representing the mRNAs was compared to that of the GAPDH mRNA band in the same lane and shown as the resultant ratio. Values are mean±SEM of three experiments. *$p<0.05$, compared with the control.