脂血症治療薬を使用していなくても血清脂質が正常化すればイベントは発生しない。（非使用群で観察期間 Y4、イベント数 D4；使用群で観察期間 kY4、イベント数 0）

Type 5（直接効果）：高脂血症治療薬を使用すると血清脂質は正常化するが、高脂血症治療薬を使わないと、血清脂質は高値のままである。薬を使った場合にはイベントが発生せず、使用しない場合にはイベントが発生する。高脂血症治療薬を使用しても仮に（事実に反して）血清脂質が正常化せず、高値のままであってもイベントは発生せず、また、高脂血症治療薬を使用していなければ仮に（事実に反して）血清脂質が正常化してもイベントは発生する。（非使用群で観察期間 Y5、イベント数 D5；使用群で観察期間 k Y5、イベント数 0）

Type6（非使用群で観察期間 Y6、イベント数 D6；使用群で観察期間 k Y6、イベント数 kD6）：治療の有無によらず、血清脂質は正常化する。血清脂質正常化しても、治療の有無によらずイベントは発生する。仮に（事実に反して）治療の有無によらず、血清脂質が高値のままでもイベントは発生する。

Type 7（直接効果）：高脂血症治療薬を使用してもしなくても血清脂質は正常化するが、薬を使った場合にはイベントが発生せず、使用しない場合にはイベントが発生する。また、仮に（事実に反して）高脂血症治療薬の治療の有無にかかわらず、血清脂質が正常化せず、高値のままであっても、薬を使った場合にはイベントは発生せず、使用しない場合にはイベントが発生する。（非使用群で観察期間 Y7、イベント数 D7；使用群で観察期間 k Y7、イベント数 0）

Type8（非使用群で観察期間 Y8、イベント数 D8=0；使用群で観察期間 k Y8、イベント数 0）：治療の有無によらず、血清脂質は高値のまま。いずれもイベント発生せず。仮に（事実に反して）治療の有無によらず、血清脂質が正常化してもイベント発生せず。

Type9（非使用群で観察期間 Y9、イベント数 D9=0；使用群で観察期間 k Y9、イベント数 0）：治療により血清脂質は正常化するが、治療しないと高値のまま。いずれもイベント発生せず。仮に（事実に反して）治療によっても血清脂質が高値のままでもイベント発生せず、あるいは仮に（事実に反して）非治療後に血清脂質が正常化してもイベント発生せず。

Type10（非使用群で観察期間 Y10、イベント数 D10=0；使用群で観察期間 k Y10、イベント数 0）：治療の有無によらず、血清脂質は正常化。いずれもイベントは発生しない。仮に（事実に反して）血清脂質が正常化せず高値のままなら治療の有無によらずイベントは発生する。

Type11（非使用群で観察期間 Y11、イベント数 D11=0；使用群で観察期間 k Y11、イベント数 0）：治療の有無によらず、血清脂質は正常化。いずれもイベントは発生しない。仮に（事実に反して）血清脂質が正常化せず高値のままでもイベント発生せず。

3. 共変量による調整方法について

本報告書作成段階においては、共変量に関する情報は得られておらず、共変量によって調整した結果を報告することはできない。また、間接効果と直接効果を求めるために最適な共変量の調整方法については、平成 17 年度報告書では十分考察されていない。

そこで本報告書では、観察研究から因果関係を推定する際の共変量の扱いに関する最近の文献[2-5]に関する文献的考察を通じて、本研究に

おける共変量の取扱いについて考察した。結果は「C. 研究結果」の「2」として示す。

C. 研究結果

1．粗発生率を用いた解析

粗発生率で検討した結果を表1に示す。

無治療群におけるイベント発生率（λ_0）はIHD9.2、CVD9.2、両者を合わせた複合イベント 18.6/1000 人-月、スタチンによる治療群におけるイベント発生率は（λ_1）はIHD6.1、CVD6.7、複合イベント 12.9/1000 人-月であった。OE（"Overall Effect"）は、IHD3.1、CVD2.5、複合イベントで5.7(95%CI:−1.6〜13.0)/1000人-月であった。OEの信頼区間はいずれも広く、信頼区間の中に0を含む。したがって直接効果と間接効果に関する解析結果の前提となる「イベント発生予防の(overallの)効果があった(なかった)」を明確に結論することはできなかった。式15、16及び式21、22で推定した間接効果と直接効果の推定値も表1に示した。CVDについては、式15、式16で推定した結果（直接効果が間接効果より大きい）と式21、22で推定した結果（間接効果が直接効果より大きい）は逆転している。ただし、いずれも信頼区間は広く0を含む。IHDに関しては、信頼区間が広い点は同じだが、おおむね間接効果より直接効果が大きいという結果であり、IHDとCVDを合わせた複合イベントについても、OEの値5.7/1000 人-月に占める間接効果の大きさは−0.3（式15）ないし0.3（式21)と推定されるのに対し、直接効果は6.0（式16）ないし5.3（式22）/1000 人-月であり直接効果が間接効果より大きいと推定された。ただし、複合イベントについても、OE、直接効果、間接効果ともに95%信頼区間は広く、いずれも信頼区間の中に0を含み、明確な結論は得られなかった。

表1. 粗発生率に基づく解析

	IHD	CVD	IHD+CVD
D10(人)	5	5	10
D11(人)	4	5	9
D1 (人)	9	10	19
Y10 (人-月)	841	871	836
Y11 (人-月)	637	626	632
Y1 (人-月)	1477	1497	1468
D00(人)	17	21	38
D01(人)	17	13	30
D0 (人)	34	34	68
Y00 (人-月)	2140	2087	2120
Y01 (人-月)	1544	1603	1531
Y0 (人-月)	3684	3690	3651
λ10 (/人月)	5.9	5.7	12.0
λ11 (/人月)	6.3	8.0	14.2
λ1 (/人月)	6.1	6.7	12.9
λ00 (/人月)	7.9	10.1	17.9
λ01 (/人月)	11.0	8.1	19.6
λ0 (/人月)	9.2	9.2	18.6
Overall effect (/人月)	3.1	2.5	5.7
(95%CI)	−1.9〜8.2	−2.6〜7.7	−1.6〜13.0
間接効果 (/人月)式15	1.1	−1.8	−0.3
(95%CI)	−3.3〜5.5	−6.3〜2.7	−6.7〜6.1
直接効果 (/人月)式16	2.0	4.3	6.0
(95%CI)	−4.4〜8.4	−2.3〜10.9	−3.4〜15.3
間接効果 (/人月)式21	−1.6	2.4	0.3
(95%CI)	−7.5〜4.3	−3.7〜8.5	−8.2〜8.9
直接効果 (/人月)式22	4.7	0.1	5.3
(95%CI)	−3.4〜12.8	−8.1〜8.4	−6.3〜17.0

2. 共変量の「調整」方法について

本報告書における間接効果と直接効果の推定方法はRobins & Greenlandが1992年に発表した「発生割合」に関する間接効果と直接効果の推定方法[1]を「発生率（/人-月)」に拡張したものである。Robinsらはその後、曝露「有」の確率を曝露以外の因子を説明変数として推定する「傾向スコア」(propensity score、PS）を用いて「事実に反する仮定」で得られるであろうリスク差、リスク比、オッズ比などを推定する「IPTW (inverse probability of treatment of weighting)」法や「SMR (standard mortality ratio) 法」などに関する一連の論文[2,4,5]を発表している。

一般に曝露群と非曝露群においてイベント発生のリスク因子の分布が異なると、これが交絡因子として働き、見かけ上ゆがんだ結果をひきおこすためこれをIPTW法やSMR法を含む何らかの方法でこれを調整する必要があるが、IPTW法とSMR法はいずれも傾向スコア(PS)

を用いる方法であり、PS の分布が等しい薬使用群と非使用群を仮想的に構成して比較をする。

この仮想的な集団（"Pseudopopulation"）[4]の構成方法は以下の通りである。たとえばPS=0.15～0.25（特定の交絡因子の組み合わせを持ち、曝露「有」の確率が 0.2 前後）である者が曝露群、非曝露群あわせて 100 人いると仮定する。PS はほぼ 0.2 に等しいので、そのうち実際に曝露「有」は 20 人程度で曝露「無」は 80 人程度と推定される。IPTW 法では実際に曝露「有」あるいは「無」の集団（それぞれ 20 人、80 人）を、PS を用いてあらわされるその曝露の状態の確率（曝露「有」群では PS (0.2)、曝露「無」群では（1-PS)(0.8)）で割る。この結果、いずれも、全コホート内に含まれる該当の PS（0.2 前後）をもつ仮想的集団（100 人）が構成される。これに対し、SMR 法では、実際に曝露「有」あるいは「無」の集団をその曝露の状態を受ける確率で割り、曝露「有」の確率すなわち PS をかける（上の例では曝露「有」群では PS（0.2）で割り PS（0.2）をかけ、曝露「無」群では（1-PS)(0.8)で割り PS（0.2）をかける)。この場合、曝露「有」群は実際に観察された集団そのもの（上記の例では 20÷0.2×0.2＝20 人)、曝露「無」群では曝露群と同じ大きさの集団が仮想的に構成される（上の例では 80÷（1－0.2）×0.2＝20 人)。

本研究で間接効果と直接効果を推定するためには、「高脂血症治療薬（スタチン以外の高脂血症治療薬を使用している者は少数であるため、実際上はスタチンによる治療）」の有無および「血清脂質高値」の有無の組み合わせからなる 4 種類の「曝露」の状態を想定すべきであろう。4 種類の曝露の確率を求めるために、まず、曝露の有無についてだけの確率を求める。即ち、全対象者から得たデータを以下のロジスティックモデルで解析する

$$\text{logit pr}[E=1 \mid L=l]=a_0+a_1 l \qquad (31)$$

ここで、L は説明変数の血清脂質以外の因子（年齢、性別、糖尿病罹患の期間、合併症など）の組み合わせである列ベクトルであり、a_1は未知のパラメータの行ベクトルである。式 31 を使って推定されたパラメータ値を$\hat{a}_0$、$\hat{a}_1$とする

さらに、曝露群のデータを用い、曝露「有」（E=1）の条件下で血清脂質高値（C=1)の確率を以下のロジスティック回帰モデルで求める。

$$\text{logit pr}[C=1 \mid L=l, E=1]=a_{01}' +a_{11}' l \qquad (32)$$

また、非曝露群のデータを用い、曝露「無」（E=0)の条件下で血清脂質高値（C=１）の確率を以下のロジスティック回帰モデルで求める。

$$\text{logit pr}[C=1 \mid L=l, E=0]=a_{00}' +a_{10}' l \qquad (33)$$

式32または式33で推定されたパラメータ値を$\hat{a}'_{0E}$、$\hat{a}'_{1E}$とする(E=0, 1)。

ある交絡因子の組み合わせをもつ曝露の状態(E,C)の傾向スコア PS(E,C)は

$$PS(E,C) = p_1^{\,E}(1-p_1)^{1-E}\, p_{2E}^{\,C}(1-p_{2E})^{1-C} \qquad (34)$$

で与えられる。ここで

$$p_1 = \frac{\exp(\hat{a}_0 + \hat{a}_1 l)}{1+\exp(\hat{a}_0 + \hat{a}_1 l)} \qquad (35)$$

$$p_{2E} = \frac{\exp(\hat{a}'_{0E} + \hat{a}'_{1E} l)}{1+\exp(\hat{a}'_{0E} + \hat{a}'_{1E} l)} \quad (E=0, 1) \qquad (36)$$

である。

式 15、16 を用いて間接効果と直接効果を推定する場合には、“Pseudopopulation” を血清脂質の高い曝露「有」の集団と同一の PS の分布をもつ集団とし、重み w に

$$w=PS(1,1)/PS(E,C) \qquad (37)$$

を用い、式 21、22 を用いて間接効果と直接効果を推定する場合には、“Pseudopopulation” を血清脂質の低い曝露「有」の集団と同一の PS の分布をもつ集団とし、重み w に

$$w=PS(1,0)/PS(E,C) \qquad (38)$$

を用いる。

D. 考察

本研究は「血清脂質管理値達成によるイベント発症予防」の効果を明らかにすることを目標に行われたコホート研究である。粗発生率を用いた解析結果は表 1 に示す通りであり、OE（“overall effect”）は IHD、CVD、IHD+CVD でそれぞれ 3.1、 2.5、5.7/1000 人-月（それぞれ 37、 30、68/1000 人-年)と推定された。また、おおむね間接効果より直接効果が大きいと推定された。しかし、その結果から単純に「スタチン使用に伴う IHD または CVD 発生の予防の効果は、血清脂質値を正常化させることを介した間接効果よりもそれ以外の血管壁などに対する直接効果によるところが大きい」と結論することには慎重でなければならない。

第一に、直接効果と間接効果に分離する以前の OE の 95%信頼区間に 0 が含まれていることに示されるように、「予防効果があった」という結論自体が現時点では明確になっていない。最低でも本研究の 2 倍程度の総観察期間が必要であり、本研究の場合、4 年程度まで観察期間を延長しない限り、信頼に足る OE の推定は困難である。

第二に、現時点では交絡因子に関する情報が得られておらず、交絡因子で調整した結果については不明である。

第三に、本研究で得られる交絡因子について調整をしても十分適切な調整は困難である可能性がある。SMR 法などで調整後の式 15、16 または式 21、22 で推定した値が適正な値であるためには、本研究で観察した対象患者の観察開始時点における曝露の有/無（E=1/0）と血清脂質高値/正常(C=1/0)を十分正確に予測することが可能な因子が本研究の研究開始時のデータで得られることが必要である。

最近の Marginal Structural Model などに関する研究では、たとえ、重要な交絡因子が全て観察されているとしても、時間とともに変動する交絡因子（たとえば血清脂質値）がイベント発生のリスク因子であり、かつ、その値がそれに引き続く曝露（ここでは高脂血症治療薬の使用）の有無に影響を与え、かつ、その交絡因子（血清脂質値）が過去の曝露（ここでは、過去の高脂血症治療薬の使用歴）によって影響を受ける場合には、現時点（あるいは、最近の）交絡因子のみで調整しても、バイアスを生ずる可能性があることが指摘されている。このような場合には、治療開始時以後の治療歴と、曝露因子の変遷のあり方全てが現時点におけるイベント発生のリスクとその後の曝露の有無に影響を与えるとする解析が必要である[4,5]。

本研究では、対象患者のほとんどは糖尿病治療開始以後、相当年数を経過しており、詳細な治療歴と交絡因子の変遷の記録は存在しないし、それらを用いた解析も不可能である。

ただし、観察開始時の曝露と血清脂質値以外の交絡因子で調整して得られる OE や間接効果、直接効果の値が、粗発生率で推定される値とどの程度異なるかなどを検討しておくことは、粗発生率から推定された OE、直接効果、間接効果の解釈にあたって有用な示唆を与えると考えられる。

また、これらの値の解釈にあたって、少なくとも OE の 95%信頼区間が 0 を含まない程度の

観察期間であることは、結果が確率誤差による可能性を否定する上で重要である。この意味で本研究の研究期間を最低限4年間程度まで延長することは重要である。

E.結論

粗発生率を用いた「血清脂質管理値達成によるイベント発症予防」効果をスタチン使用によるIHDとCVDの予防効果（OE）を、血清脂質の正常化（LDL値120mg/dL以下）によってもたらされる「間接効果」とそれ以外の「直接効果」に分離することで評価することを試みた。粗発生率で解析する結果を見る限り、スタチンによるイベント予防効果には「直接効果」が相当程度関与していることが示唆されたが、また、薬使用と血清脂質以外の危険因子に関するデータが得られておらず、結論は今後に待たなければならない。またサンプルサイズが小さく、最低限4年間程度まで観察期間を延長することが望ましい。

F. 健康危険情報

特記すべきものなし.

G. 研究発表

1．論文発表：なし

2．学会発表：なし

H. 知的財産権の出願，登録状況：なし

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Ⅲ. 研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別冊

Endothelial cellular senescence is inhibited by nitric oxide: Implications in atherosclerosis associated with menopause and diabetes

Toshio Hayashi*†, Hisako Matsui-Hirai*, Asaka Miyazaki-Akita*, Akiko Fukatsu*, Jun Funami*, Qun-Fang Ding*, Sumitra Kamalanathan*, Yuichi Hattori‡, Louis J. Ignarro†§, and Akihisa Iguchi*

*Department of Geriatrics, Nagoya University Graduate School of Medicine, Tsuruma-cho 65, Showa-ku, Nagoya 466-8550, Japan; ‡Department of Pharmacology, School of Medicine, University of Toyama, Sugitani 2630, Toyama 930-0194, Japan; and §Department of Molecular and Medical Pharmacology, Center for the Health Sciences, David Geffen School of Medicine, University of California, 650 Charles E. Young Drive-South, Los Angeles, CA 90095-1735

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Senescence may contribute to the pathogenesis of atherosclerosis. Although the bioavailability of nitric oxide (NO) is limited in senescence, the effect of NO on senescence and its relationship to cardiovascular risk factors have not been investigated fully. We studied these factors by investigating senescence-associated β-galactosidase (SA-β-gal) and human telomerase activity in human umbilical venous endothelial cells (HUVECs). Treatment with NO donor (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO) and transfection with endothelial NO synthase (eNOS) into HUVECs each decreased the number of SA-β-gal positive cells and increased telomerase activity. The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) abolished the effect of eNOS transfection. The physiological concentration of 17β-estradiol activated hTERT, decreased SA-β-gal-positive cells, and caused cell proliferation. However, ICI 182780, an estrogen receptor-specific antagonist, and L-NAME each inhibited these effects. Finally, we investigated the effect of NO bioavailability on high glucose-promoted cellular senescence of HUVECs. Inhibition by eNOS transfection of this cellular senescence under high glucose conditions was less pronounced. Treatment with L-arginine or L-citrulline of eNOS-transfected cells partially inhibited, and combination of L-arginine and L-citrulline with antioxidants strongly prevented, high glucose-induced cellular senescence. These data demonstrate that NO can prevent endothelial senescence, thereby contributing to the anti-senile action of estrogen. The ingestion of NO-boosting substances, including L-arginine, L-citrulline, and antioxidants, can delay endothelial senescence under high glucose. We suggest that the delay in endothelial senescence through NO and/or eNOS activation may have clinical utility in the treatment of atherosclerosis in the elderly.

diabetes mellitus | endothelial nitric oxide synthase | estrogen | aging

Aging is known to be a major cardiovascular risk factor (1). Cellular senescence is the limited ability of human cells to divide when cultured *in vitro* and is usually accompanied by phenotypic changes in morphology, gene expression, and function (2). These changes have been implicated in human aging. Until recently, little attention has been paid to the potential impact of vascular cellular senescence on age-related vascular disorders. Senescent cells from aged animals express increased levels of proinflammatory molecules, suggesting that cellular senescence *in vivo* contributes to the pathogenesis of human atherosclerosis (3).

The telomere hypothesis is a widely accepted explanation of the occurrence of senescence (4). Telomeres, repetitive DNA sequences at the ends of eukaryotic chromosomes, shorten as a linear function of increasing cellular division, and according to the hypothesis, short telomere length triggers the onset of senescence (5, 6). Telomerase, a ribonucleoprotein, can synthesize new telomeric repeats and restore telomere length. Cellular senescence usually accompanies telomere shortening and increases in senescence-associated β-galactosidase (SA-β-gal) (assayed at pH 6), which is distinguishable from endogenous lysosomal β-gal activity, is considered to be a marker of cellular senescence (7).

According to a free-radical theory, reactive oxygen species (ROS) may be potential candidates responsible for senescence, and oxidative stress may promote senescence by shortening telomere through inactivation of the Src kinase family (8–10). Therefore, not only atherosclerosis but also senescence has been shown to progress via ROS (11). NO is a widespread signaling molecule in the cardiovascular system, which functions in multiple ways to protect against the initiation and progression of atherosclerosis (12, 13). NO prevents the adhesion and aggregation of blood cells and inhibits vascular smooth muscle cell proliferation (14). However, neither the role nor the effect of endothelial NO on senescence is fully known. As NO can abrogate the state of oxidative stress, NO may thus have the potential to affect cellular senescence by scavenging senescent stimuli such as ROS.

Accordingly, the present study was performed to examine whether or not NO and the activation of eNOS can delay endothelial senescence. We also considered estrogen depletion and diabetes mellitus among various cardiovascular risk factors as applied models of the combined effects of NO on both atherosclerosis and cell longevity.

The morbidity of cardiovascular disease dramatically increases after menopause (15). In such cases, estrogen depletion has been speculated as a cause of the disease, and estrogen plays an antiatherogenic role both *in vivo* and *in vitro* (16, 17). Although hormone replacement therapy was reported not to prevent cardiovascular disease in a clinical trial, this ineffectiveness was due to the increased frequency of thrombosis produced by estrogen in advanced atherosclerosis and to the adverse effect of coprescribed progesterone (18). The fact that females are known to live several years longer than males world-wide strongly supports the antiatherogenic effect of estrogen.

Diabetes mellitus is also a major cardiovascular risk factor, and the etiology of diabetic atherosclerosis is suggested to include the increase of ROS and the decrease of NO bioavailability as a result

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Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SA-β-gal, senescence-associated β-galactosidase; hTERT, human telomerase reverse transcriptase; HUVECs, human umbilical vein endothelial cells; L-NAME, N^G-nitro-L-arginine methyl ester; DETA-NO, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl) amino] diazen-1-ium-1,2-diolate; PDL, population-doubling level.

†To whom correspondence may be addressed. E-mail: hayashi@med.nagoya-u.ac.jp or lignarro@mednet.ucla.edu.

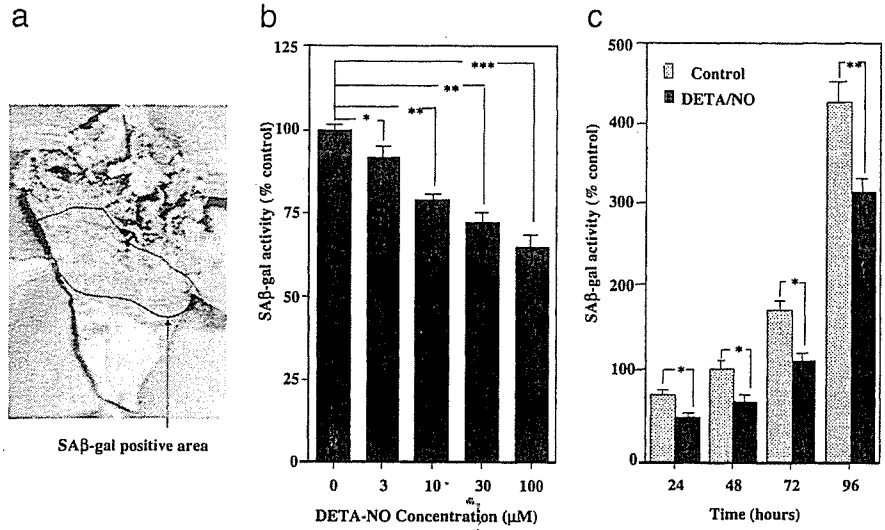

Fig. 1. SA-β-gal activity as cellular senescence. (*a*) SA-β-gal-positive staining was observed in atherosclerotic lesions of the intimal side of human thoracic aorta, which was obtained by autopsy. No staining was detected in the nonatherosclerotic area and advanced atherosclerotic area, including the necrotic core and ulcer complicated lesion. (*b*) Concentration-dependent decrease in SA-β-gal activity in HUVECs by DETA-NO. HUVECs were treated with DETA-NO for 24 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ vs. DETA-NO-untreated control. (*c*) Time-dependent decrease in SA-β-gal activity in HUVECs by DETA-NO. HUVECs were treated with 10 μM DETA-NO for 24–96 h. *, $P < 0.05$; **, $P < 0.01$ vs. the corresponding control. Control sample, which is treated for 48 h, is expressed as 100%.

of high glucose levels (19). The incidence of cardiovascular diseases is increased in elderly diabetic patients, but the relationship between senescence and diabetes on endothelial function has yet to be elucidated (20). NO is synthesized by NOS, which utilizes L-arginine as a substrate and produces L-citrulline as the second reaction product. L-arginine can be synthesized from L-citrulline in endothelial cells through a recycling pathway (21). This pathway may be the principal mechanism for sustaining localized L-arginine availability for endothelial nitric oxide synthase (eNOS)-catalyzed NO production (21, 22). In the present study, we examined the effect of NO boosting on high glucose and/or senescence by the regulation of eNOS.

Results

NO Delays Cellular Senescence. The effect of NO on endothelial cellular senescence was investigated by evaluating SA-β-gal used as a cellular senescence marker and human telomerase activity used as an indicator of elongation of telomere length in human umbilical vein endothelial cells (HUVECs). We also examined SA-β-gal activity in the thoracic aorta and coronary arteries obtained from 3 autopsied elderly individuals. Fig. 1*a* shows that SA-β-gal activity was observed in the mild atherosclerotic area in human thoracic aorta. Treatment with the NO donor, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-aminoethyl) amino] diazen-1-ium-1,2-diolate (DETA-NO), for 24 h significantly decreased the SA-β-gal activity in HUVECs (Fig. 1 *b* and *c*). The effect of DETA-NO was found to be both concentration-dependent (3–100 μM, Fig. 1*b*) and time-dependent (24–96 h treatment, Fig. 1*c*). Coincubation with N^G-nitro-L-arginine methyl ester (L-NAME) (300 μM) did not affect the action of DETA-NO (data not shown). DETA-NO also increased telomerase activity in HUVECs (data not shown).

Transfection with eNOS into HEK 293 cells or HUVECs for 48 h increased the NO metabolite, $NO2^-$ (Fig. 2*a*), and also significantly increased telomerase activity (Fig. 2*b*). On the other hand, the number of SA-β-gal-stained cells was reduced by eNOS transfection (data not shown). Fig. 2*c* shows the effects of eNOS-related substrate and products on SA-β-gal staining in HUVECs. Coincubation with the NOS inhibitor L-NAME (300 μM) tended to decrease the number of SA-β-gal-stained cells by inhibiting NO release from HUVECs. SA-β-gal-positive staining also tended to decrease in the presence of L-arginine and/or L-citrulline. However, their effects on SA-β-gal-stained cells are not statistically significant even though they were given together.

Estrogen Delays Cellular Senescence via an NO-Dependent Mechanism. We next investigated the effect of E2 on cellular senescence in HUVECs. At physiological concentrations (10 nM), E2 treatment reduced the number of SA-β-gal-positive cells, especially in large population-doubling level (PDL) cells (Fig. 3*a*). Fig. 3*b* shows representative photographs of SA-β-gal-stained cells in HUVECs of PDL 22. E2 decreased the number of SA-β-gal-stained cells, whereas this effect was prevented by coincubation with L-NAME (Fig. 3*b*). E2 markedly activated telomerase, and this activation was inhibited by ICI 182780, an estrogen receptor-specific antagonist, and by L-NAME (Fig. 3*c*). These results suggest that the counteracting effect of E2 on senescence involves an eNOS-dependent mechanism by means of activation of estrogen receptors.

The physiological concentration of E2 also enhanced proliferation of HUVECs (Fig. 4). As the HUVEC proliferating activity tended to slow down in senescent cells, this basal mechanism seems to be different from that underlying the effect of E2 on telomerase and SA-β-gal. On the other hand, L-NAME treatment decreased proliferation of HUVECs in all PDL (Fig. 4*a*). The peak effect on cell proliferation was achieved with physiological concentrations of E2, whereas higher E2 concentration produced a lesser effect (Fig. 4*b*).

The Effect of NO Bioavailability on High-Glucose-Induced Cellular Senescence. Finally, the effects of NO bioavailability on cellular senescence under high glucose conditions were investigated. Exposure to high glucose for 24 h decreased the expression level of eNOS protein in a manner dependent on the concentration of glucose, resulting in decreases of 19% at 11 mM and 33% at 22 mM glucose compared with the control (5.5 mM glucose) level (Fig. 5*a*). Mannitol, used as an osmolarity control, had no influence on eNOS protein level. In HUVECs cultured under high glucose conditions (11, 22, and 31 mM) for 3 days, nitrite ($NO2^-$) production was decreased (Fig. 5*b*) and intracellular ROS production was increased (data not shown) in a manner dependent on the concentration of glucose. Treatment with L-arginine, L-citrulline, and antioxidants (vitamin C and E) alone or in combination showed a significant recovery of the decreased nitrite level under high glucose condi-

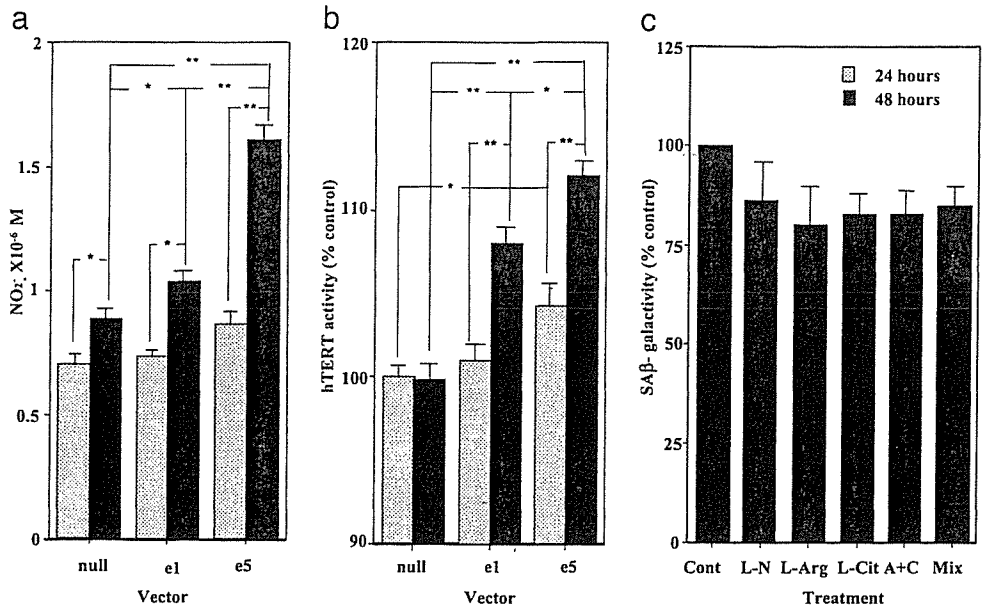

Fig. 2. Influence of eNOS modulation on cellular senescence. (*a*) The effect of transfection with eNOS on nitrite production by HEK 293 cells. Transfection with eNOS into cells was performed; e5 included five times the amount of eNOS vector compared with e1. The nitrite concentrations in the medium 24 and 48 h after transfection are shown. *, $P < 0.05$; **, $P < 0.01$. (*b*) The effect of transfection with eNOS on telomerase activity in HEK 293 cells. The activity of hTERT in cells 24 and 48 h after transfection are shown. *, $P < 0.05$; **, $P < 0.01$. (*c*) The effects of treatment with L-NAME (L-N, 300 μM), L-arginine (L-arg, 1 mM), and L-citrulline (L-cit, 300 μM) alone or in combination (A+C) on SA-β-gal activity in HUVECs. The treatment time was 24 h. Mix = L-arginine, L-citrulline and vitamin E plus vitamin C (each, 100 μM).

tions (Fig. 5*c*). When L-arginine, L-citrulline and antioxidants were given together, the recovery of nitrite production was more marked.

High glucose exposure for 72 h promoted cellular senescence as indicated by increases in SA-β-gal-positive staining (Fig. 6) and decrease in telomerase activity (data not shown). The number of SA-β-gal-positive staining cells under high glucose conditions tended to decrease slightly after incubation with L-arginine, L-citrulline, and antioxidants alone, and was significantly decreased when they were given together (Fig. 6 *a* and *b*). Moreover, transfection with eNOS tended to prevent cellular senescence slightly, and the combined presence of L-arginine, L-citrulline, and antioxidants very effectively prevented it under high glucose conditions (Fig. 6*c*).

Discussion

The free-radical theory of aging proposes that degenerative senescence is largely the result of the cumulative effect of ROS (9, 10). It is possible that some association exists between increased oxidative stress and reduced telomerase activity. Interestingly, individuals with shorter white blood cell telomeres tend to show a >2.8-fold higher coronary risk than the highest quartile for telomere length, after adjusting for age (23).

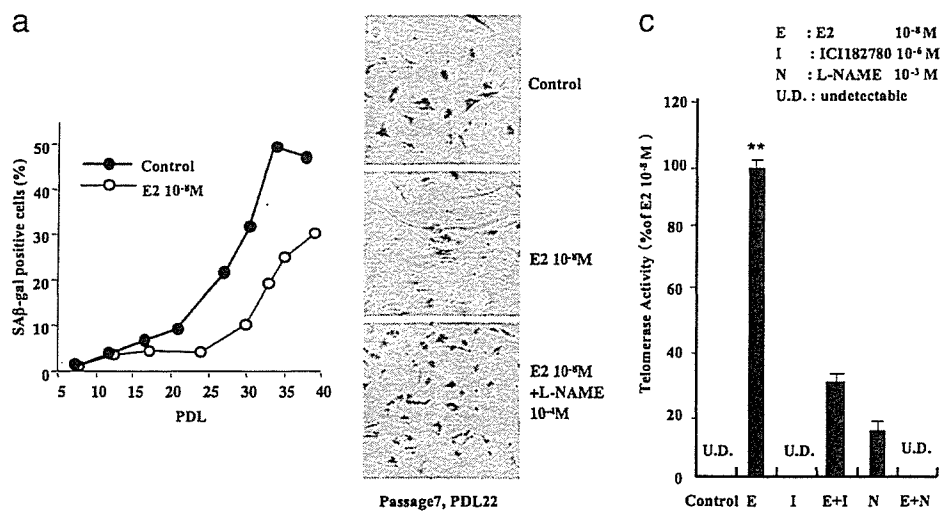

Fig. 3. Effect of estrogen on cellular senescence. (*a*) The relative levels of SA-β-gal-positive staining cells in different PDL when HUVECs were untreated and treated with 10^{-8} M E2 for 24 h. Positive staining cells were evaluated by FACscan. (*b*) Representative photographs of SA-β-gal staining in control, 10^{-8} M E2-treated, and 10^{-8} M E2- and 10^{-4} M L-NAME-treated cells. Note that treatment with E2 decreased the number of SA-β-gal-positive cells, which was prevented by further treatment with L-NAME. Cells were used in PDL 22 at passage 7. (*c*) The effects of E2 (E, 10^{-8} M), ICI 182780 (I, 1 μM), and L-NAME (N, 1 mM) on telomerase activity in HUVECs. UD, undetectable. **, $P < 0.01$ vs. control.

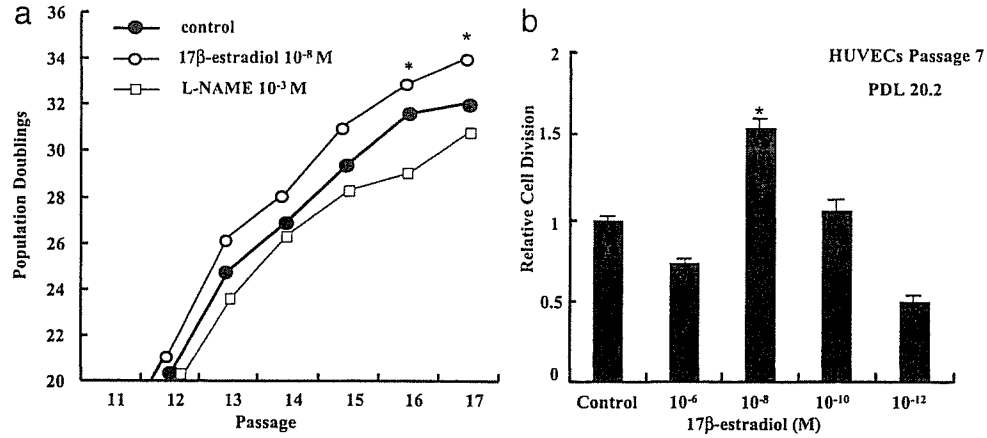

Fig. 4. Effects of E2 on endothelial cell proliferation. (*a*) The effects of E2 (10^{-8} M) and L-NAME (1 mM) on population doublings in each passage of HUVECs. The treatment time with E2 or L-NAME was 24 h. *, $P < 0.05$ vs. control. (*b*) The effects of different concentrations of E2 on relative cell division of HUVECs. Cells were used in PDL 20.2 at passage 7. *, $P < 0.05$: cell division vs. control.

Telomerase counteracts the shortening of telomeres and contains a catalytic subunit, the hTERT (4, 5). The introduction of hTERT into human cells extends both their lifespan and their telomeres to lengths typical of those of young cells (5, 6). The regulation of hTERT involves both transcriptional and posttranscriptional mechanisms. Transcriptional regulation is believed to be the main regulatory mechanism in cancer cells (24). Telomerase activity can be posttranscriptionally regulated by kinases such as protein kinase C (PKC), extracellular signal-regulated kinase 1/2 (ERK1/2), and Akt [Akt/PKB (protein kinase B)] in endothelial cells (8–10). ROS formation leads to an increase in Src-family kinase activation and a reduction of Akt expression in aging endothelial cells. It is speculated that phosphorylation by Akt keeps hTERT in an active status in the nucleus, whereas increasing the activation of Src-family kinases induces the nuclear export of hTERT, thereby reducing the ability to lengthen telomeres and protect from aging. Along with the enhanced ROS formation, we found that a decrease in telomerase activity preceded the onset of replicative senescence. Thus, ROS such as the superoxide radical and H_2O_2, which are formed during aerobic metabolism, are generally considered to be important regulators of the aging processes, and their production may be mainly due to the actions of NADPH oxidase and the mitochondria (9, 10, 24–26). In the present study, we showed that DETA-NO, an NO donor, and eNOS transfection activate hTERT and increase scavenging of ROS. L-NAME inhibited the effect of eNOS transfection. These results mean that telomerase activity was likely regulated by NO bioavailability. Our data indicated that eNOS transfection has comparable effects to hTERT transfection on both cellular aging and telomerase activity. In addition, these findings might also indicate that endothelial cell aging is linked to the balance between ROS formation and NO bioavailability, which in turn affects telomerase activity.

eNOS transfection has an antiatherosclerotic effect even in cases of advanced atherosclerosis, and the administration of L-arginine with the gene transfer of eNOS enhances the effect of eNOS transfection (27, 28). We showed that the coadministration of antioxidants with L-arginine and L-citrulline produces an enhanced antiatherosclerotic response in advanced atherosclerosis (29). L-arginine seems to increase the production of NO, whereas antioxidants most likely protect the newly formed NO against destruction by ROS. Recent evidence indicates that the bulk of intracellular endothelial L-arginine may not be available for NO production, because intracellular L-arginine for eNOS may be limited by uptake into plasmalemmal caveolae (30). The pathway by which L-citrulline is recycled to L-arginine is localized to the caveolae and it may be the main source of available L-arginine (21, 22, 29, 31). L-citrulline is converted to L-arginine by mammalian cells, including endothelial cells. This recycling pathway might, therefore, play an important role in sustaining the production of NO in endothelial cells by providing available L-arginine, especially in advanced atherosclerosis or diabetes mellitus, when plasma L-arginine levels are depleted.

Physiological concentrations of E2 activate telomerase activity and decrease the number of SA-β-gal-stained cells through the

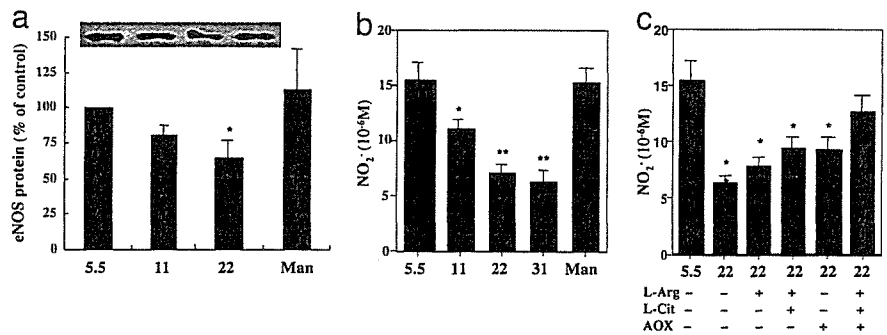

Fig. 5. Influence of high glucose on eNOS expression and nitrite production. (*a*) The effect of exposure to different concentrations of glucose on the level of eNOS protein expression in HUVECs. Mannitol (Man) was given as an osmolarity control. Cells were kept under different glucose conditions for 72 h. *, $P < 0.05$ vs. normal (5.5 mM) glucose. (*b*) The effect of exposure to different concentrations of glucose on nitrite levels in culture medium of HUVECs. *, $P < 0.05$; **, $P < 0.01$ vs. normal glucose. (*c*) The effects of L-arginine (L-arg, 1 mM), L-citrulline (L-cit, 300 μM), and antioxidants (AOX, 100 μM vitamin E plus 100 μM vitamin C) alone or in combination on nitrite levels in culture medium in HUVECs, which were reduced by 22 mM glucose. *, $P < 0.05$; **, $P < 0.01$ vs. normal glucose.

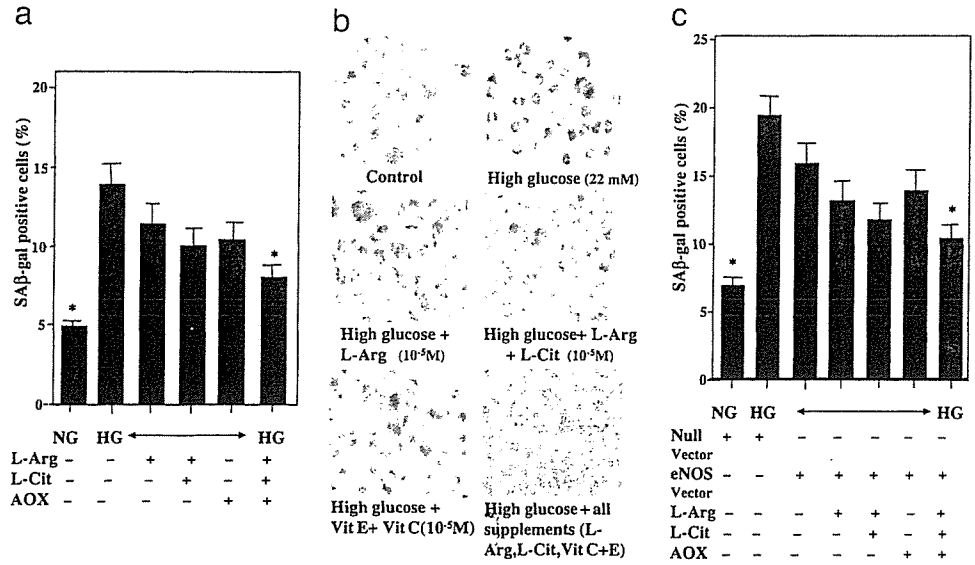

Fig. 6. Influence of high glucose for 72 h on cellular senescence of HUVECs. (*a*) The effects of L-arginine (L-arg, 1 mM), L-citrulline (L-cit, 300 μM), and antioxidants (AOX, 100 μM vitamin E plus 100 μM vitamin C) on the increase in β-gal-positive stained cells when exposed to high (22 mM) glucose. *, $P < 0.05$ vs. high glucose without any treatment. (*b*) Representative photographs showing cellular senescence by staining cells with SA-β- gal. (*c*) Modulation by transfection with eNOS of the effects of L-arginine, L-citrulline, and antioxidants on the increase in SA-β-gal-positive-stained cells when exposed to high glucose. Null vector is control vector of eNOS Vector. *, $P < 0.05$ vs. high glucose without any treatment. NG, normal glucose; HG, high glucose (22 mM).

estrogen receptor and NO-dependent mechanisms. E2 treatment also stimulated the proliferation of HUVECs through the estrogen receptor and NO-dependent mechanisms. We reported the possibility that such effects of estrogen were mediated by the direct effect on eNOS and the scavenging effect on ROS-producing enzymes such as NADPH oxidase, especially the p22phox subunit (32, 33). It is, therefore, proposed that estrogen exerts its effect on endothelial cell senescence by increasing NO bioavailability, which may then reduce ROS generation and subsequently prevent the nuclear export of TERT.

Atherosclerosis is an inflammatory disease characterized by endothelial dysfunction, impairment of NO production (1, 12 13), and oxidative stress (11), which can lead not only to cell membrane injury but also to the destruction of NO. Diabetic macroangiopathy occurs under almost the same conditions, with increased levels of superoxide from NADPH oxidase and impairment of NO production (34, 35). In the present study, high-glucose-induced endothelial dysfunction, oxidative stress, and cellular senescence were reversed with the administration of L-arginine, L-citrulline, and antioxidants. A lack of GTP cyclohydrolase I, which is the rate-limiting enzyme of tetrahydrobiopterin (BH4) synthesis, a cofactor of eNOS, also reduces NO production (36). We speculate that not only BH4 but also L-arginine, L-citrulline, and antioxidants are important in diabetic macroangiopathy. Although NO is known to be involved in reducing both oxidative stress and the progression of atherosclerosis, the present study also assessed the consequence of the NO-mediated delay of cellular senescence on the progression of atherosclerosis. The aforesaid notwithstanding, the local expression (bioavailability) of NO remains an important factor in the maintenance of normal tissue function. We also cannot exclude the possibility that other factors than NO is involved in the progressive cellular senescence in diabetes.

Taken together, the present data provide evidence demonstrating an NO-dependent mechanism in the delay of endothelial cell senescence. Consequently, the antiatherosclerotic action of NO is particularly profound under conditions of aging, estrogen depletion, and diabetes mellitus. NO could, therefore, scavenge the age-associated increase in ROS and thereby reduce the coronary risk factor-induced increase in ROS. Moreover, our data indicate that NO may also prevent endothelial cell senescence, possibly by interfering with the redox balance of endothelial cells.

Methods

Materials. We used 17β-estradiol (Sigma, St. Louis, MO), D-glucose, D-mannitol (Wako, Osaka, Japan), Takara One Step RNA PCR Kit (Takara, Kyoto, Japan), and eNOS monoclonal antibody (BD Biosciences, San Jose, CA). ICI 182780 was kindly provided by Zeneca Pharmaceuticals. L-NAME and DETA-NO were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies to β-galactosidase (Chemicon International, Lexington, NY) were used (7, 37).

Cell Culture. HUVECs were purchased from Clonetics (San Diego, CA) and cultured in low-glucose EBM-2 supplemented with 10% calf serum, EBM-2 including EGM-2 SingleQuots (Clonetics), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air. These cells were positive for the endothelial cell-specific von Willebrand factor and angiotensin-1-converting enzyme activity. The cells were seeded into six-well plates, and subconfluent cell monolayers were studied within six to eight passages. Before starting the experimental procedures, the medium was removed and replaced with phenol red-free low-glucose D-MEM supplemented with 1% calf serum, 0.06% glutamine, and 1% penicillin–streptomycin. In some experiments accompanying eNOS transfection, HEK 293 cells were treated instead of HUVECs because of relative ease of transfection. The rate of PDL was calculated at each passage until growth arrest based on the following formula: $PDL = (\log_{10}Y - \log_{10}X)/\log_{10}2$ (Y indicates the number of cells counted at the end of the passage; X is the number of cells seeded). Cumulative population doubling was calculated as the sum of all of the changes in population doubling.

Measurement of Nitrite. The methods for measuring nitrite (NO_2^-) production by HUVECs have been previously described by our laboratory. In brief, samples of the incubation culture medium were recovered after centrifugation to remove any precipitated materials. The nitrite concentrations of the supernatants were determined by high-performance liquid chromatography (ENO10; EICOM,

Kyoto, Japan) as described (29, 38). The incubated medium was not completely free of nitrite; therefore, an aliquot of medium was assayed by the same process as the medium obtained from the cultured cells. We used the nitrite value obtained in the medium alone as a blank, and it was subtracted from all of the samples.

Flow Cytometric Analysis of ROS Generation. The determination of intracellular oxidant production in HUVECs was based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCFDA) resulting in the formation of the fluorescent compound 2′,7′-dichlorofluorescein (DCF) (Molecular Probes, Eugene, OR) (29, 38). Carboxy-H2DCFDA freely diffuses across the cell membrane, is diacylated, and incorporates into hydrophobic lipid regions of the cell. HUVECs were incubated at 37°C for 30 min in PBS in which 2 μl of 5 mM H2DCFDA was added. After incubation, the dye was aspirated and the cells were trypsinized and washed once by centrifugation at 1,670 $\times$ *g* for 5 min to remove trypsin and extracellular H_2DCFDA. HUVECs were resuspended in PBS and transferred into 5 ml of polystyrene round-bottom tubes with cell-strainer caps (Becton Dickinson, Franklin Lakes, NJ). They were protected from light and kept cold until ready for analysis on a FACS caliber flow cytometer (Becton Dickinson) set at ≈515- to 545-nm excitation. The emission filters used a 530/30-nm bandpass.

SA-β-Gal. HUVECs and tissues were fixed and stained for SA-β-gal activity as described (37). In brief, the cells were fixed for 10 min in 2% formaldehyde, 0.2% glutaraldehyde in PBS, and incubated for 12 h at 37°C without CO_2 with fresh β-gal staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$, pH 6.0. The cells were counterstained with 4′6-diamidinophenylindole (DAPI; 0.2 mg/ml in 10 mM NaCl) for 10 min to count the total cell number. The percentage of SA-β-gal-positive cells was determined by counting the number of blue cells within a sample of 1,000 cells. We also used the Flow Cytometric Analysis.

Human Telomerase Activity. The quantitative determination of telomerase activity was performed according to the manufacturer's protocol for the TeloTAGGG telomerase PCR ELISAPLUS Kit (Roche Diagnostics, Mannheim, Germany) based on the telomeric repeat application protocol (TRAP) assay. To measure telomerase activity, 2 μg of protein was used in the PCR.

Western Blot Analysis of eNOS. Total protein was extracted from the endothelial cells and then analyzed by Western blotting (38, 39). Briefly, the protein concentration was determined with a Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples of cell homogenate (5 μg) were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to poly(vinylidine difluoride) filter membranes. To reduce any nonspecific binding, the membrane was preincubated for 30 min at room temperature in TTBS (150 mM NaCl/10 mM Tris, pH 8.0/0.05% Tween 20) containing 5% nonfat milk. The membrane was then incubated overnight with the primary antibody at 3:10,000 dilutions in PBS (0.075 μg/ml). The membrane was incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 60 min at room temperature. The blots were washed in TTBS and subsequently visualized with the aid of a SuperSignal West Dura Trial Kit (Pierce Biotechnology, Rockford, IL), exposed to x-ray film, and analyzed by the NIH Image Software program produced by Wayne Rasband (National Institutes of Health, Bethesda, MD). Loading of equal amounts of protein was confirmed by Coomassie brilliant blue and Amido black staining of protein in each lane of the same blot.

Construction of an Adenovirus Vector Carrying eNOS and Transfer into Cultured ECs. Recombinant adenoviruses containing eNOS cDNA were constructed by using the ADENO-QUEST Kit (Quantum, Quebec City, Canada) (27). Briefly, bovine eNOS cDNA (provided by T. Michel, Harvard University, Cambridge, MA) was cloned into the AdBM5pAG vector. The resulting plasmid was then cotransfected with viral DNA into HEK 293 cells. We incubated 5×10^5 HUVECs in a six-well plate for 24 h, then incubated cells with adenoviruses at a multiplicity of infection of 20 for 24 h. For all of the studies, the viral titers were adjusted to 2×10^9 pfu/ml. Adenoviruses carrying an *Escherichia coli lacZ* gene encoding a nucleus-localized variant of β-gal (Ad. β-gal) or no cDNA (Ad.-null) were also used. We also used eNOS/pcDNA3.1(+) and Qiagen Effectane Transferase Reagent.

Statistics. All data are given as means ± SEM from at least three independent experiments. Comparisons between the two groups were made based on the nonparametric Mann–Whitney *U* test. Statistical significance was evaluated with repeated-measures ANOVA by using a least-significant difference (LSD) post hoc test or ANOVA for multiple comparisons (SPSS Software 11.0). Differences were considered to be significant at a value of $P < 0.05$.

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Nitric oxide (NO) is a new clinical biomarker of survival in the elderly patients and its efficacy might be nearly equal to albumin

Masako Osawa [a], Toshio Hayashi [a,*], Hideki Nomura [a], Jun Funami [a], Asaka Miyazaki [a], Louis J. Ignarro [b], Akihisa Iguchi [a]

[a] *Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya City 466-8550, Japan*
[b] *Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, 650 Charles E. Young Drive South, Los Angeles, CA 90095, USA*

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Abstract

Background: For elderly patients, the consideration of prognostic factors is very important, but there have been few reports about the potential use of vasoactive substances as prognostic markers in the elderly.

Objective: We assessed endocrinological substances, such as plasma NO_x (metabolites of NO), as the prognostic marker in elderly. We compared their efficacy with that of such well-known markers as albumin and pro-inflammatory cytokines such as IL-6.

Methods: The patients were recruited consequently from the clinics of Nagoya University Hospital or related home care services facilities. One hundred and twenty seven elderly aged 65 and older were registered. Biochemical analyses such as albumin, total cholesterol, BNP, and NO_x were measured upon enrollment. The main outcome was the survival rate.

Results: Forty-six patients died during the follow-up period. Mann–Whitney's *U*-test showed that the levels of age, hemoglobin, total protein, serum albumin, serum creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, high sensitive CRP, NO_x, IL-6, and TNF-α were significantly different between the living and deceased subjects. Among the dependent variables in the logistic regression analyses, only albumin and NO_x were significantly different. In the Kaplan–Meier analyses of mortality, the prognosis of patients in 3rd and 4th quartile of NO_x was significantly worse than that in 1st or 2nd quartile.

Conclusion: NO_x has potential both as a vascular marker and as a marker for predicting survival in elderly. In the latter role, it may be as effective as albumin.

Keywords: Nitric oxide; cGMP; Albumin; Biomarker; Elderly; Prognostic marker; Vascular functional marker

Many nations, including Japan, are experiencing rapid growth in their elderly populations. The main causes of death in Japanese elderly are heart disease, cerebro-vascular disease, and cancer. Several biochemical markers, such as albumin and cholesterol, have been identified as having prognostic value for mortality and hospitalization [1–3]. Recent studies also have indicated the potential role of the immune system in the pathophysiology of congestive heart failure (CHF) and malignancy [4,5]. Plasma levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) also have been reported to be significant prognostic predictors in patients with CHF or malignancy) [6–8]. TNF-α induces adhesion molecule expression such as ICAM-1 on endothelial cells, which promotes the progression of atherosclerosis [9]. In other words, in older populations, peripheral blood markers of nutrition or inflammation (albumin, cholesterol, IL-6, and TNF-α) have been individually shown to be increased risk for mortality [2,10,11].

In elderly people, the rate of CHF is important for predicting mortality and hospitalization rates. Brain natriuretic peptide (BNP) is a good marker of CHF, because the plasma BNP concentration is elevated according to the severity of CHF [12–15]. Binding of BNP to its receptors

* Corresponding author. Fax: +81 52 744 2371.
E-mail address: hayashi@med.nagoya-u.ac.jp (T. Hayashi).

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initiates natriuretic and vasorelaxant activities through an elevation in intracellular cyclic guanosine monophosphate (cGMP) [16,17]. Nitric oxide (NO) is also an important vasoactive substance, because it exerts anti-atherogenic effects by inhibiting the migration or proliferation of monocytes or smooth muscle cells and vasodilation mainly by cGMP dependent mechanism [18]. We reported that NO regulates cGMP in patients with renal insufficiency [19]. NO may be a useful prognostic marker for patients suffering from atherosclerotic diseases such as cerebral strokes or myocardial infarction, although as yet there have been no reports investigating the use of NO in this capacity. The source of NO is not only endothelial cells (endothelial NO synthase; eNOS) but also macrophages or T cells (inducible NO synthase; iNOS) and some neuronal cells (neuronal NO synthase; nNOS). The plasma level of NO_x (nitrite plus nitrate, metabolites of NO) may reflect the status of eNOS and, to some extent, the status of iNOS. Because iNOS is activated in patients with inflammations such as sepsis, advanced stages of malignancy, or progressed atherosclerotic lesions, the NO_x level may have potential as a marker of malignancy as well as atherosclerotic diseases [20,21].

For elderly patients, the consideration of prognostic factors is very important, but there have been few reports about the potential use of vasoactive substances. Therefore, in this study, we evaluated whether measurements of plasma levels of vasoactive factors such as NO_x, cytokines such as IL-6, and well-known markers such as albumin were useful as prognostic factors in the elderly.

Methods

Study sample

One hundred and twenty seven elderly subjects (48 males and 79 females; mean age, 81.3 ± 7.5 years; range, 65–101 years) were enrolled on August on 2002. The study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and written informed consent was obtained from all patients. Patients were selected consecutively among our geriatric clinics and related home care services. In brief, 91 participants were presented at Department of Geriatrics, Nagoya University Hospital and the related hospital as outpatients (31 from their homes, 31 from geriatric nursing care units, and 29 from other facilities such as private homes for the aged) and 36 were in home care services facility. At the baseline examination, participants underwent a review of their medical history, a physical examination, and assessment of cardiovascular disease risk factors. On registration, they were not suffering with acute or evident heart failure or acute inflammation whose serum CRP is larger than 2 mg/dl. They were also not suffering with acute myocardial infarction or cerebral infarction within 3 months. We followed patients up to 2.8 years. All participants had a clinical visit each year of the study period, and their laboratory data were determined at each of these visits. We had telephone contact with the patients who could not have clinical visit, or their physicians.

Measurement

We measured fasting serum or plasma levels of biochemical products including lipids and plasma levels of neurohumoral factors and cytokines. Levels of general biochemical products were measured at SRL Laboratories, Tokyo, on an automated sequential multiple analyzer. Samples for the assay of plasma norepinephrine (NE), angiotensin-II, BNP, NO_x, cGMP, IL-6, and TNF-α levels were transferred to chilled disposable tubes containing EDTA-2Na. The blood samples were immediately placed on ice and centrifuged at −4 °C, and aliquots of plasma were immediately stored at −80 °C until assay. BNP levels were measured with a specific radioimmunoassay. NE levels were measured by HPLC. NO_x levels were measured using an NO detector-HPLC system (ENO10; Eicom Co., Kyoto, Japan) [22]. cGMP concentration was determined using a specific radioimmunoassay method (RPN226; Amersham, Buckinghamshire, England) [23]. Angiotensin-II levels were measured by radioimmunoassay. Both IL-6 and TNF-α measurements were performed using a commercially available radioimmunoassay kit (Quantikine HS; R&D Systems, Minneapolis, MN). Hypertension was defined as systolic BP ≥ 140 mmHg, or diastolic BP ≥ 90 mmHG or antihypertensive drugs were prescribed. Hyperlipidemia was defined as follows. Total cholesterol ≥ 220 mg/dl or LDL cholesterol (total cholesterol − HDL cholesterol − triglyceride/5) ≥ 140 mg/dl) or anti-hyperlipidemic drugs were prescribed. Diabetes mellitus was defined as in American Diabetes Society Guidelines [24] (in brief, fasting blood glucose ≥ 126 mg/dl or hemoglobin A1C ≥ 6.5 g/dl). Previously diagnosed hypertension, hyperlipidemia or diabetes were also included.

Statistical analysis

The results are presented as means ± SD. Values of $P < .05$ were considered to indicate statistical significance in all analyses. All statistical analyses were performed using Stat View software (SAS Institute Inc., Cary, NC). Characteristics of the survivors and the deceased subjects were compared using Mann–Whitney's *U*-test. Characteristics that were significantly different between the survivors and deceased by Mann–Whitney's *U*-test were further subjected to inherent multiple logistic regression analysis. As a result, adjusted odds ratios were calculated. Survival curves were calculated by the Kaplan–Meier method.

Results

Clinical characteristics

Table 1 shows the baseline characteristics of patients. There were no significant differences in age or coronary risk factors among the situations where the patients were

Table 1
Baseline characteristic of patients on registration

Characteristics $n = 127$			
Profiles		Chronic medications	
Age (years old)	81.31 ± 7.48	Angiotensin converting enzyme inhibitor	24
Male	48	Angiotensin II receptor antagonist	18
Female	79	β-Blocker	9
		Ca antagonist	37
Medical history			
Hypertension	57	Diuretics	29
Hyperlipidemia	27		
Diabetes mellitus	36	Nitrate	29
		Digitalis	16
Prior congestive heart failure	24		
Ischemic heart disease	21	Antiplatelet agents	49
Arrhythmia	23	Warfarin	8
Stroke	51	Statins	16
Arteriosclerosis obliterans	6		

enrolled. Their complicating diseases were hypertension (44.9%), hyperlipidemia (21.3%), diabetes mellitus (28.3%), congestive heart failure (18.9%), ischemic heart disease (16.5%) and cerebral vascular disease (40.2%, including patients with only lacuna). Table 1 also shows the chronic medications of patients. Anti-hypertension drugs ⟨angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blocking agents (ARBs), β-adrenergic receptor blocking agents and calcium antagonists⟩, diuretics, antiplatelet agents, and nitrates [isosorbide dinitrate 20 mg/day, $n = 14$, 40 mg/day $n = 3$, nitroglycerin patch 25 mg/day $n = 12$] were prescribed frequently.

Survival

During the 2.8-year follow-up period, 46 of the 127 patients died (Table 2). Seventeen, seventeen, and twelve patients died during the 1st, 2nd, and 3rd year of the follow-up period, respectively. The causes of death were as follows: cardiovascular disease ($n = 15$ patients: 6 cases of heart failure, 3 of acute myocardial infarction, 4 of renal failure related to cardiovascular disease, and 2 of cerebro-vascular disease), infection ($n = 13$), malignancy ($n = 2$), cardiopulmonary arrest without evident cause ($n = 6$), liver cirrhosis ($n = 1$), intestinal twist ($n = 1$), dehydration ($n = 1$), senility ($n = 1$), burned to death ($n = 1$), and unknown ($n = 5$). Four patients cannot be followed by medical record or telephone contacting. There are no significant differences in gender ratio (male/female) between participants being alive after 2.8 years and those dead during 2.8 years. There are also no significant differences between them in the drugs or the suffered diseases including ischemic heart disease, congestive heart failure, and smoking.

Table 2
Biochemical characteristic of patients on registration

	On registration ($n = 127$)	Participants being alive after 2.8 years ($n = 77$)	Participants dead during 2.8 years ($n = 46$)	*P* value
Hemoglobulin (g/dl)	11.6 ± 1.9	12.2 ± 1.7	10.7 ± 1.8	.0001
Total protein (g/dl)	7.04 ± .71	7.15 ± .69	6.84 ± .69	.0129
Albumin (g/dl)	3.83 ± .52	3.98 ± .42	3.56 ± .55	.0001
Total cholesterol (mg/dl)	197.8 ± 46.0	210.6 ± 45.3	178.1 ± 41.4	.0003
HDL-cholesterol (mg/dl)	50.1 ± 20.5	53.2 ± 20.1	44.6 ± 19.7	.0078
LDL-cholesterol (mg/dl)	123.8 ± 35.2	131.9 ± 35.9	111.7 ± 31.7	.0038
Triglyceride (mg/dl)	113.3 ± 62.3	114.6 ± 55.7	111.9 ± 74.4	.2577
Creatinine (mg/dl)	.88 ± .6	.80 ± .38	.94 ± .69	.4518
cGMP (pmol/ml)	4.89 ± 3.2	4.74 ± 3.2	5.40 ± 3.0	.1114
Hs-CRP (ng/ml)	8224.9 ± 17224	6493.1 ± 15669	11123.7 ± 19391	.0150
HANP (ng/ml)	43.1 ± 56.3	38.1 ± 36.7	52.5 ± 79.4	.2673
BNP (ng/ml)	79.1 ± 81.1	71.3 ± 81.6	92.6 ± 77.9	.0565
NO_x (μmol/l)	28.3 ± 19.3	25.1 ± 18.4	33.7 ± 19.8	.0059
Norepinephrine	541.0 ± 439.7	493.0 ± 431.7	619.2 ± 478.4	.3103
Angiotensin II	9.03 ± 8.90	8.74 ± 7.78	9.50 ± 10.57	.7047
Il-6 (pg/ml)	7.81 ± 10.0	7.17 ± 9.6	8.99 ± 11.0	.0174
TNF-α (pg/ml)	1.94 ± 6.021	2.177 ± 7.7	1.58 ± .89	.0001

Values are expressed as means ± SD. $P < .05$: significant difference between alive and dead. Hs-CRP: high sensitive C reactive protein, HANP: human atrial natriuretic peptide, BNP: brain natriuretic peptide. NO_x: NO metabolites.

Biochemical analyses

The biochemical characteristics of all participants are shown in Table 2. The age, hemoglobin, albumin, total cholesterol, HDL cholesterol, and LDL cholesterol levels on registration were significantly higher in those who survived than in those who died during the study period. Gender or creatinine was not significant in this study.

Markers profile

The plasma concentrations of the various makers are also depicted in Table 2. Plasma NO_x and IL-6 were significantly lower in those who were alive after the study period than in those who died during the period. Plasma TNF-α was also higher in the survivors than in the patients who died. BNP level on registration was not different significantly ($P = .0565$).

Multivariate survival analyses

Logistic regression was applied to analyze significant characteristics: age, hemoglobin total protein, albumin, total cholesterol, HDL-cholesterol, LDL-cholesterol, IL-6, TNF-α, and NO_x, which showed significant differences between the survivors and the deceased by using Mann–Whitney's *U*-test, which was described as above. They were subjected to logistic regression analysis. The results are depicted in Table 3. The adjusted odds ratios of albumin and NO_x were .236 and 1.027, respectively. Based on this result, 1SD decrease of albumin level from a certain level is estimated to be 2.11 in odds ratio. 1SD increase of NO_x level from a certain levels is to be 1.67 in odds ratio. As the effect of albumin and that of NO_x are independent, the odds ratio increases synergistically. NO_x was shown to correlate the serum hemoglobin, HDL-cholesterol, creatinine, and HS-CRP on baseline level, and NO_x level was known to be affected by age, gender, and creatinine (renal function). So, we thought creatinine and gender as independent risk factor, and re-applied logistic regression including creatinine and gender as well as the conditions above. The result is almost same as before that albumin and NO_x are significant, and that the adjusted odds ratios of albumin and NO_x were .195 ($P = .045$) and 1.031 ($P = .034$) respectively.

Table 3
Multivariate logistic regression analysis

	Adjusted odds ratio	95% CI	*P* value
Age (year)	1.047	.977–1.122	.194
Hemoglobulin (g/dl)	.766	.562–1.044	.0917
Total protein (g/dl)	.997	.458–2.170	.9932
Albumin * (g/dl)	.236	.058–.955	.0429
Total Cholesterol(mg/dl)	.994	.949–1.042	.8083
HDL-Cholesterol (mg/dl)	1.000	.948–1.055	.9972
LDL-Cholesterol (mg/dl)	.993	.943–1.046	.7988
Hs-CRP (ng/ml)	1.001	1.000–1.002	.0924
NO_x * (μ mol/l)	1.027	1.010–1.541	.0394
Il-6 (pg/ml)	.975	.904–1.051	.5072
TNF-α (pg/ml)	.967	.727–1.288	.8197

Values are expressed as means ± SD. $P < .05$: significant difference between alive and dead. Hs-CRP: high sensitive C reactive protein, HANP: human atrial natriuretic peptide, BNP: brain natriuretic peptide, NO_x: NO metabolites.
CI: confidence interval. * means the significant marker ($P < .05$).

These data showed that NO is a useful prognostic marker with efficacy almost equal to that of albumin, the best-known prognostic marker to date. BNP and other cytokines such as IL-6 or TNF-α were not significant prognostic markers in the present study. Further, in the patient who died by cardiovascular diseases, multivariate survival analyses showed that NO_x and the history of ischemic heart disease were significant prognostic markers. In patients who died by other causes than cardiovascular diseases, NO_x and albumin were significant markers.

Survival rate

The survival rates are shown in Fig. 1. Panel (A) shows the relation between survival rate and NO_x levels on entry, and panel (B) shows the relation between survival rate and albumin levels on entry. The survival rate goes up in proportion to NO_x levels and goes down in proportion to albumin levels. Kaplan–Meier analyses of mortality show that the prognosis of patients was dependent on NO_x levels. The prognosis of patients in 3rd and 4th quartile of NO_x was significantly worse than that in 1st or 2nd quartile of NO_x. The prognosis of patients in first quartile of albumin is worse than that of other patients, which there is no difference in prognosis between 2nd, 3rd or 4th quartile of albumin. In other words, NO_x levels reflect the viability of all patients; however,in albumin level, the patients in 4th quartile only have worst prognosis.

Discussion

The present data demonstrate that NO is a new clinical biomarker of survival in elderly patients and that its efficacy might be nearly equal to that of albumin, the best known prognostic marker to date. This is the first report of the use of a vascular functional marker such as NO as a prognostic marker in the elderly.

We assessed for around 3 years prognosis to expect short and mid term prognosis. Because elderly patients might have many illnesses—e.g., occult congestive heart failure, arteriosclerosis, latent malignancy, etc.—we evaluated nutrition markers, pro-inflammatory cytokines, natriuretic peptide and vascular endocrinological substances.

Serum albumin and cholesterol levels were reported as prognostic marker and it was reported recently that elderly individuals with low cholesterol constitute a heterogeneous group with regard to health characteristics and mortality risk [1–3]. It is needless to say that inflammatory markers or cytokines are valuable, but these substances are often

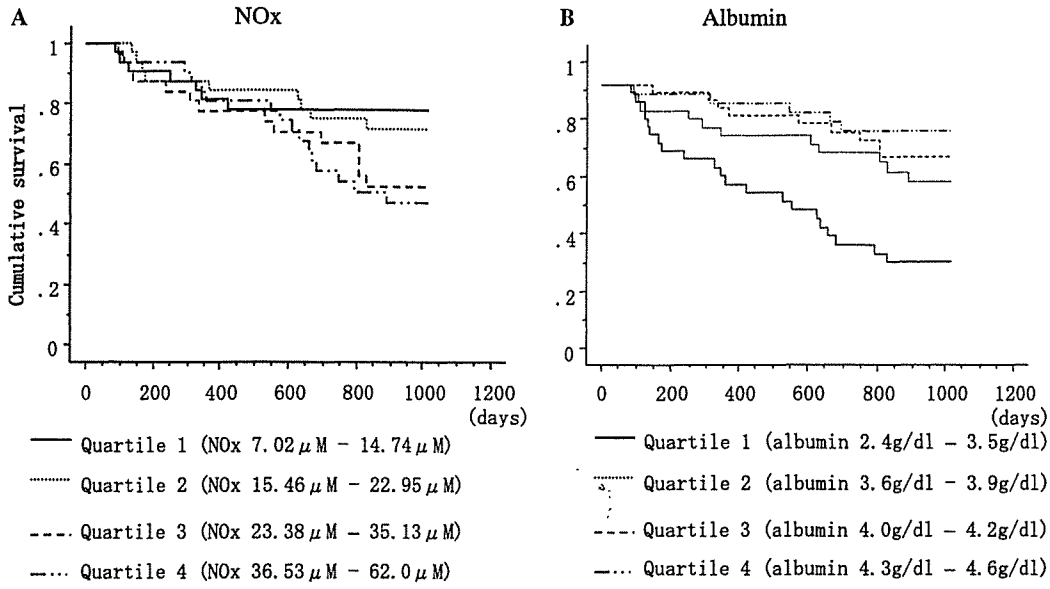

Fig. 1. Kaplan–Meier survival analysis. Circulating levels of NO_x (A) and albumin (B) were examined in relation to patient survival during follow-up. For this analysis, circulating levels of NO_x and albumin were arbitrarily divided into quartiles on registration.

affected by acute infections or chronic collagen disease such as rheumarthritis, which is very common in elderly people.

The present study revealed that low albumin, low cholesterol, high IL-6 and high sensitive-CRP indicate poor prognosis. However, logistic regression analysis indicated that only albumin and NO_x were predictive.

NO is produced by L-arginine and NO synthases (eNOS in endothelial cells, iNOS mainly in inflammatory cells, and nNOS in the nervous system). Atherosclerosis is an inflammatory disease [25] characterized by vascular endothelial cell dysfunction and diminished production of NO [26]. eNOS gene transfer can reduce atherogenesis in hypercholesterolemic animals [27]. NO is a widespread signaling molecule in the cardiovascular system, and functions in multiple ways to protect against the progression of atherosclerosis [28–30]. Plasma NO_x was difficult to assay and estimate, because protein of plasma affects the NO_x value. HPLC developed for measurement of NO_x specially is convenient and seemed to be accurate and reliable in those points. Plasma concentrations of NO_x [31] are higher in patients with CHF, and NO_x concentrations may vary according to the severity of heart failure [32]. Both the natriuretic peptide family and NO mediate their physiological action through a second messenger, cGMP [33–35]. NO increases production of cGMP by activation of soluble guanylate cyclase [33–35], while the natriuretic peptide family increases production of cGMP by activation of particle guanylate cyclase. In the present study, NO_x increased not only in the patients with heart failure but also in the patients with other atherosclerotic diseases.

In animal experiments including ours, the mRNA and protein of eNOS were increased in atherosclerotic vessels [36]. Further, coronary risk factors such as hyperlipidemia were also shown to increase mRNA of eNOS of vessels. In plasma drawn from vein, it is possible that NO_x amount increased because of high eNOS in vessels, although arterial endothelial dysfunction occurred because of intimal thickening and increased reactive oxygen species intra arteries. Taken together, we speculate NO_x levels reflects pre-clinical and clinical situation of both the vessels (atherosclerosis induced ischemia etc.) and heart (heart failure etc.) and thus NO_x was most sensitive in this study.

There were no patients with sepsis or advanced malignancy at registration. No patients died within the 3 months after registration, and the maximum levels of NO_x were at most 3 times the mean, indicating the less contribution of iNOS in this study and NO_x levels principally reflect eNOS derived one. In fact, we observed iNOS only in some macrophage derived foam cells and T lymphocytes in the peripheral of necrotic core of advanced atherosclerosis, not in mild or moderate atherosclerosis or vein [37]. Although the detailed mechanisms should be elucidated, a continuous increase in NO was suggested to have a deteriorating effect on the prognosis and might be used to predict the prognosis.

Study limitations

We measured the plasma NO_x level in order to assess basal NO production. We were afraid that plasma NO_x concentration might be affected by exogenous NO sources such as diet or drugs (NO donors or eNOS activator like angiotensin converting enzyme inhibitors; ACE-I) [38,39]. We collected fasting blood samples. We evaluated the effect of NO donors (nitroglycerin etc.), eNOS activators (ACE-Is etc.) and patients' diseases on plasma NO_x level. The Mann–Whitney's *U*-test showed no significant effects (ACEI .219, HMG-CoA reductase inhibitor .391, NO donors .291, ischemic heart diseases .307 etc). The effect of drugs may be masked between elderly patients because of