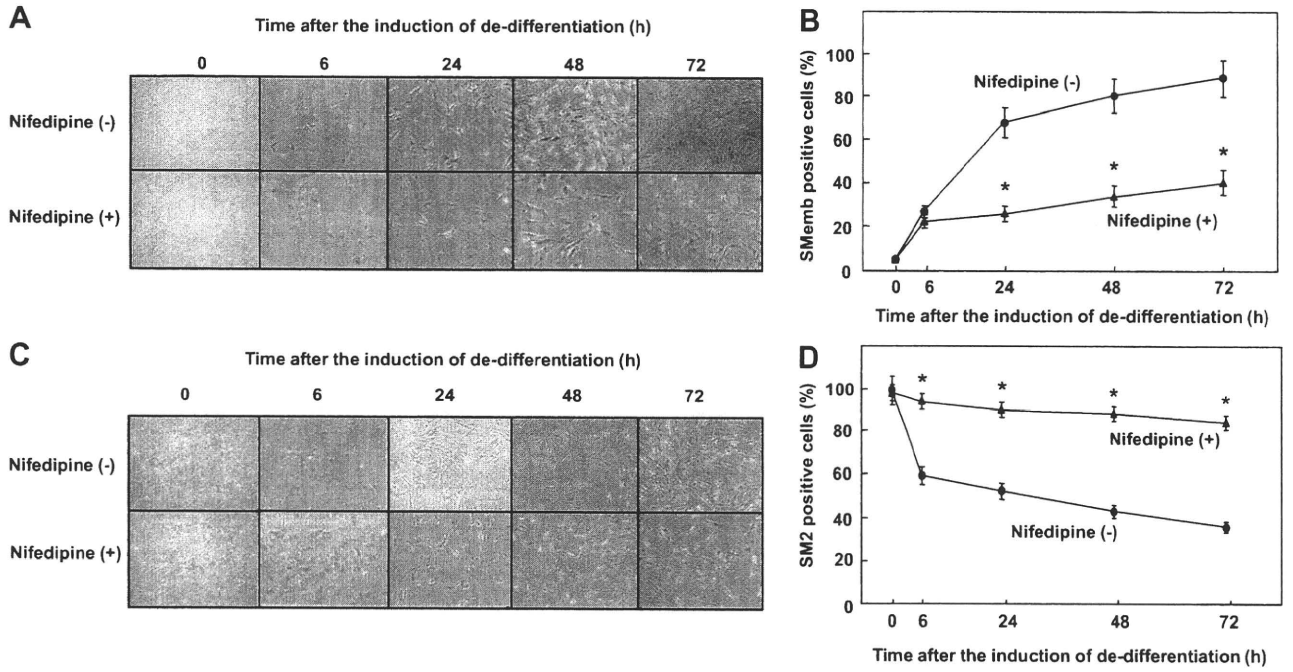

Figure 1. Nifedipine inhibits VSMC dedifferentiation in culture. Differentiated VSMCs cultured on laminin-coated dishes were transferred to laminin-uncoated dishes to induce dedifferentiation and then cultured in medium containing 5% FBS in the presence or absence of nifedipine (50 μmol/L). Cells were then immunostained with SMemb (A) and SM2 (C) at 6, 24, 48, and 72 hours after induction of dedifferentiation. Percentages of positive cells stained for SMemb (B) or SM2 (D) were counted after the immunostaining procedure. *$P<0.05$ vs VSMCs without nifedipine.

cantly inhibited both upregulation of SMemb (Figure 3A and 3B) expression and downregulation of SM2 (Figure 3C and 3D) expression induced by dedifferentiation. These findings indicate that the Akt signaling is involved in dedifferentiation of VSMCs in culture.

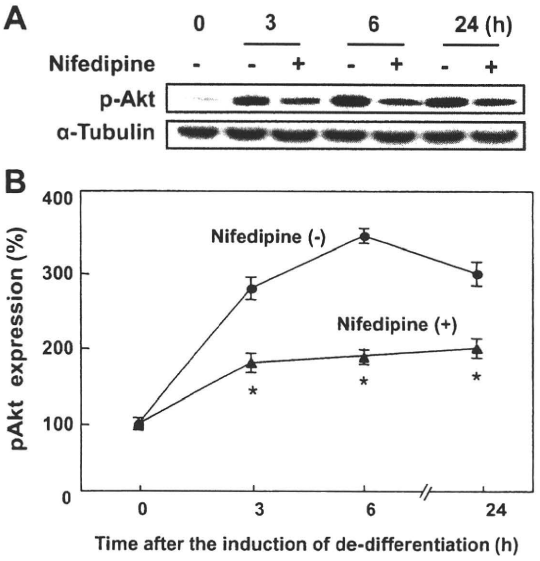

Figure 2. Nifedipine inhibits the Akt signaling upregulated by VSMC dedifferentiation. A, Phosho-Akt expression after induction of VSMC dedifferentiation with or without nifedipine (50 μmol/L) was determined by Western blot analysis. B, Phospho-Akt expression was quantified by densitometric analysis from 4 independent experiments. Data are normalized for α-tubulin and expressed as the mean±SEM (n=5). *$P<0.05$ vs VSMCs without nifedipine.

Nifedipine Inhibits the Akt Signaling in the Intimal VSMCs In Vivo

Next, we examined whether nifedipine inhibits the Akt signaling during VSMC dedifferentiation in vivo. As shown in Figure 4, phospho-Akt was highly expressed by actin-positive VSMCs in the intima. Nifedipine treatment significantly inhibited the Akt signaling in intimal VSMCs. In contrast, phospho-Akt–positive endothelial cells were present in the regenerating endothelium of nifedipine-treated arteries, suggesting that the Akt signaling in the endothelium is not affected by nifedipine.

Nifedipine and Downregulation of Akt Inhibit VSMC Dedifferentiation In Vivo

To test whether nifedipine inhibits VSMC dedifferentiation through downregulation of the Akt signaling in vivo, we evaluated the effect of nifedipine treatment and Akt gene transfer on VSMC dedifferentiation in the balloon injury model. Nifedipine treatment significantly suppressed the intimal hyperplasia (Figure 5A and 5B) and inhibited upregulation of SMemb expression and downregulation of SM2 expression in the intima (Figure 5C). Downregulation of Akt by transfer with DN-Akt was as effective as nifedipine at inhibiting hyperplasia and VSMC dedifferentiation in the intima. Conversely, upregulation of Akt by transfer with CA-Akt reversed the inhibition of VSMC dedifferentiation. These findings may provide causal evidence that the modulation of the Akt signaling is responsible for inhibiting VSMC dedifferentiation by nifedipine treatment.

Discussion

Calcium antagonists are widely used in the treatment of hypertension and angina pectoris. Recent evidence suggests

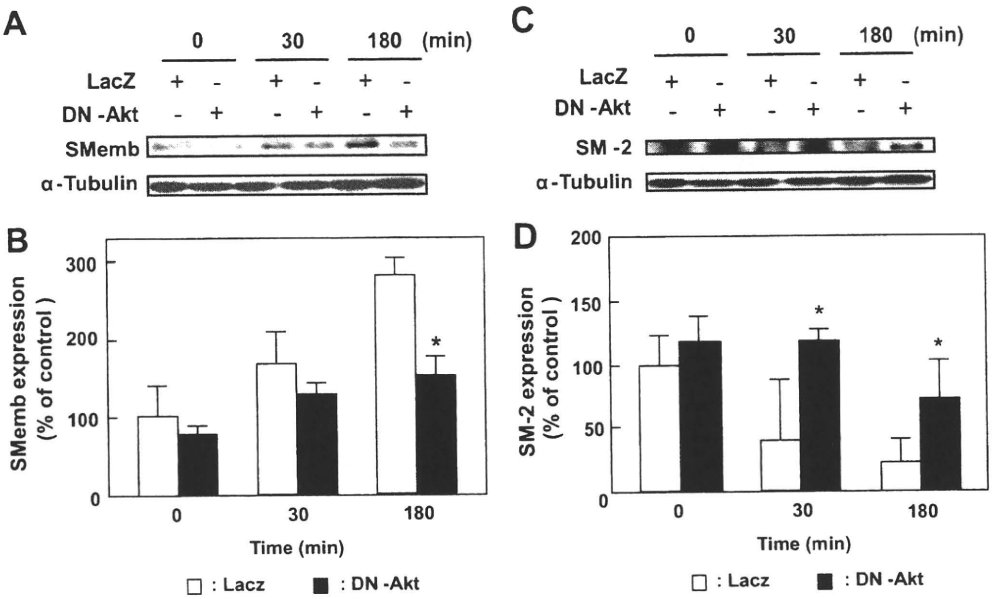

Figure 3. Downregulation of Akt inhibits VSMC dedifferentiation. Differentiated VSMCs were infected with LacZ or DN-Akt for 48 hours. VSMC dedifferentiation was induced in these cells, and VSMCs were then harvested at 0, 30, and 180 minutes after induction of dedifferentiation. SMemb expression (A) and SM2 expression (C) by were determined by Western blot analysis. Then, the blot was reprobed for α-tubulin to confirm equal loading of protein in each well. SMemb expression (B) and SM2 expression (D) were quantified by densitometric analysis from 5 independent experiments. Data are normalized for α-tubulin and expressed as the mean±SEM. *$P<0.05$ vs VSMCs treated with LacZ.

that these drugs improve the clinical outcome in patients with certain cardiovascular diseases.[20] Generally, nifedipine and other dihydropyridine derivatives are considered to retard VSMC proliferation by reducing the cellular availability of calcium and interfering with the calcium-calmodulin complex to inhibit VSMC proliferation and migration. In addition, nifedipine has been shown to modulate low-density lipoprotein metabolism by macrophages. However, it has not been clear whether the Akt signaling is involved in the beneficial effect of calcium antagonists on the development of cardiovascular diseases.

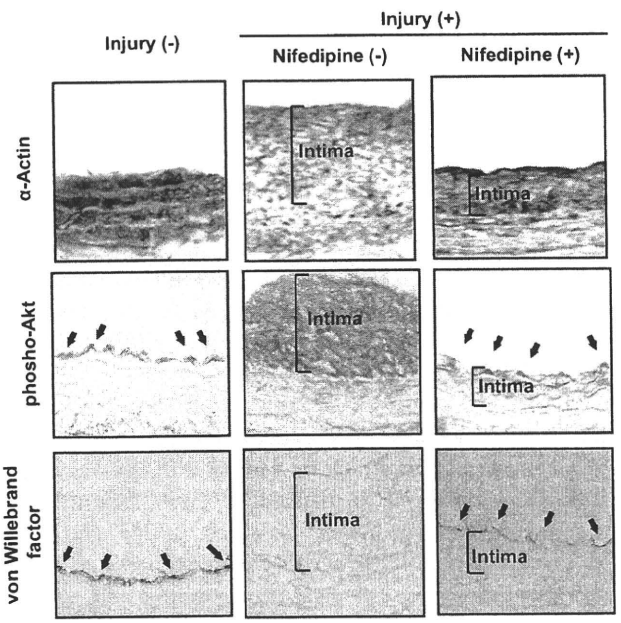

Figure 4. Nifedipine inhibits upregulation of the Akt signaling in intimal VSMCs but not in the endothelium after the rat balloon injury model. Nifedipine was administered for 3 weeks at 0.3 mg/kg per day. The balloon injury was performed 1 week after the administration of nifedipine. Expression for α-actin, phosphor-Akt, and von Willebrand factor was immunostained in injured arteries at 2 weeks after the balloon injury.

In the present study, we showed that nifedipine inhibits VSMC dedifferentiation and suppresses neointimal thickening after balloon injury. The nifedipine concentration used in this study does not reduce the blood pressure; this suggests that it has an antiatherogenic effect that is independent of its influence on blood pressure. This finding is consistent with that of studies that have reported the inhibition of atherosclerosis through a direct antioxidant effect of calcium antagonists on endothelial cells[10,11]; these studies have also reported that calcium antagonists exhibit an antiatherogenic action without causing any reduction in the blood pressure or changes in the plasma lipid profile.[21]

PDGF-BB was overexpressed at sites of VSMC proliferation after balloon injury of the vessels and at sites of atherosclerosis, suggesting its role in the development of intimal thickening. In fact, blocking of PDGF-BB or its receptor has been reported to inhibit neointimal thickening.[1] Therefore, PDGF-BB seems to be responsible for the phenotypic changes and VSMC dedifferentiation in vivo. Recently, costimulation with PDGF-BB and interleukin 1β has been proved to induce sustained activation of Akt and p70S6K.[22] In the present study, we showed that nifedipine suppresses PDGF-induced increases in phospho-Akt expression. Our preliminary experiments also showed that nifedipine inhibits increases in both phosho-Akt1 and phospho-Akt2 (data not shown). These findings suggest that nifedipine inhibits PDGF-induced neointimal thickening by suppressing Akt phosphorylation and VSMC dedifferentiation.

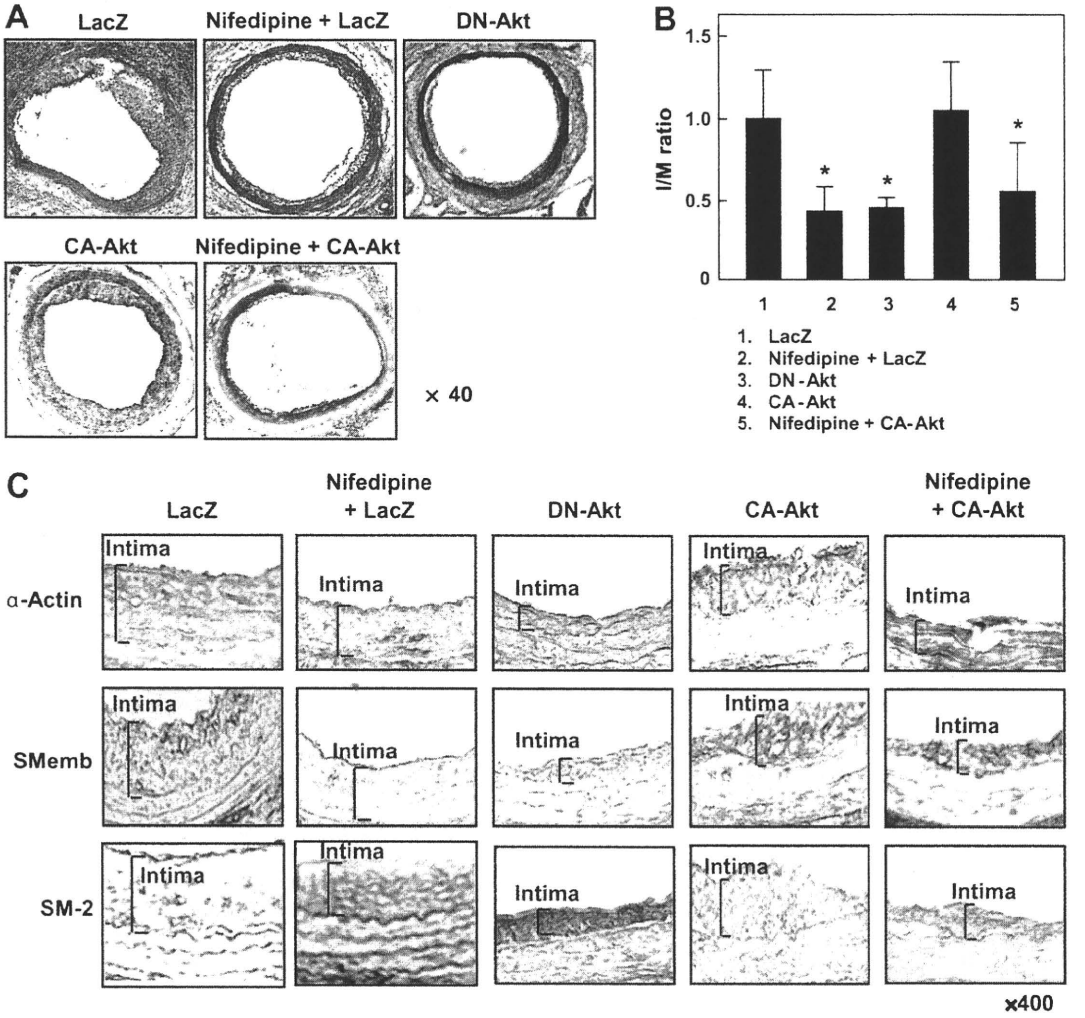

Figure 5. Nifedipine or DN-Akt transfer inhibits both VSMC proliferation and dedifferentiation in the intima after the balloon injury. Nifedipine (0.3 mg/kg per day) was administrated for 1 week before the balloon injury. DN-Akt gene transfer significantly suppressed neointimal thickening similar to nifedipine treatment. After the staining for hematoxylin-eosin (A), intima:media ratio (I/M ratio; B) was calculated (n=5 in each group). *$P<0.05$ vs the I/M ratio of the LacZ or Ad-CA-Akt groups. C, Expression levels for α-actin, SM-2, and SMemb in the intima were compared among 5 groups (LacZ, nifedipine+LacZ, DN-Akt, CA-Akt, and nifedipine+CA-Akt) after the immunostaining.

Nifedipine suppressed the increased phospho-Akt expression in vitro. Moreover, treatment of injured arteries with nifedipine reduced Akt phosphorylation in the neointimal VSMCs in vivo. These results indicate that nifedipine suppresses the activation of Akt signaling and thereby inhibits VSMC dedifferentiation. In contrast, nifedipine did not affect the Akt signaling in the endothelial cells of injured arteries. This finding is reasonable because endothelial cells are not known to have any receptors for calcium antagonists. This result is also supported by our previous finding that nifedipine indirectly enhances NO production by endothelial cells by stimulating vascular endothelial growth factor release from the VSMCs.[23] Our present findings have the important implication that regeneration of endothelial cells and upregulation of endothelial NO synthase expression via Akt signaling activated by vascular endothelial growth factor and other growth factors may not be suppressed by calcium antagonists.[24,25]

The number of functional L-type calcium channels significantly decreased in dedifferentiated VSMCs and increased on differentiation.[26] This is consistent with our finding that nifedipine inhibits the dedifferentiation of differentiated VSMCs, thereby suppressing the development and progression of atherosclerosis.

Perspectives

We demonstrated that nifedipine, an L-type calcium channel antagonist, inhibits upregulation of the Akt signaling in VSMCs but not in the regenerating endothelium. Our results also suggest that modulation of the Akt signaling by nifedipine leads to an inhibition of VSMC dedifferentiation in injured arteries. These findings may provide new insights into the mechanisms underlying the beneficial effects of calcium antagonists in the treatment of cardiovascular diseases.

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Disclosures

None.

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《原　著》

内皮細胞におけるミトコンドリア局在たん白質 Apop-1 による グルコースのミトコンドリア活性酸素種産生増大作用

河端　真実[1)]　竹村　幸宏[3)]　深田　瑠美[2)]
山本　遥菜[2)]　安田　修[4)]　福尾　惠介[1,2)]

要旨　最近，糖尿病による動脈硬化の病態にミトコンドリア活性酸素種の産生増大が関与すると報告されている．今回我々は，内皮細胞において，ミトコンドリア局在たん白質 Apop-1 がグルコースによるミトコンドリア活性酸素種の産生増大を増強する可能性を検討した．内皮細胞を高濃度グルコース処理すると，ミトコンドリアのスーパーオキシド産生増大やミトコンドリア障害が誘導された．しかし，活性酸素種消去剤 NAC の添加により，高濃度グルコース処理によるミトコンドリア障害の誘導が阻害された．また，高濃度グルコース処理をした内皮細胞で，Apop-1 たん白質発現量が増加したが，Apop-1 siRNA を導入し，内因性の Apop-1 発現を抑制すると，ミトコンドリアのスーパーオキシド産生が有意に抑制された．以上より，内皮細胞において，グルコースによるミトコンドリア活性酸素種の産生増大に，Apop-1 が関与することが示唆された．この結果から，Apop-1 は糖尿病による動脈硬化の進行に関与する新しいミトコンドリア局在たん白質であると推察された．

キーワード：Apop-1，ミトコンドリア，活性酸素種，内皮細胞

緒　言

動脈硬化の予防には，内皮細胞の機能保持が重要である[1)]．内皮細胞より産生される一酸化窒素（NO）は，動脈硬化進行の抑制に重要な役割を果たしている[2)]．しかし，動脈硬化のリスク因子である高血圧，脂質代謝異常症，または糖尿病などを有する患者の動脈では，活性酸素種が多量に産生されるため，NO 作用が不十分となり，動脈硬化進行促進をみる[3)]．

ミトコンドリアは，エネルギー代謝と関係の深い細胞内小器官であるが，活性酸素種の産生器官でもある[4,5)]．重要なことに，高血糖による内皮細胞のミトコンドリア活性酸素種の産生増大は，糖尿病による血管障害や動脈硬化の発症に関与する[6)]．しかし，高血糖がどのようなメカニズムでミトコンドリア活性酸素種の産生を増大させるかは明らかではない．

Apop-1 は，動脈硬化進行に伴って特異的に発現亢進するミトコンドリア局在たん白質である．Apop-1 は血管平滑筋細胞に過剰発現すると，ミトコンドリアのチトクローム C 遊離を促進し，アポトーシスを誘導する[7)]．今回我々は，内皮細胞においてミトコンドリア局在たん白質である Apop-1 がグルコースによるミトコンドリア活性酸素種の産生増大を増強する可能性を検討した．

方　法

1）細胞培養

ヒト臍帯静脈由来内皮細胞（HUVEC）（クラボウバイオメディカル，大阪）を用い，10% ウシ胎仔血清（FBS）を含む専用培地 HuMedia-EG 2（クラボウバイオメディカル，大阪）で培養した．

2）ミトコンドリアのスーパーオキシド産生量の測定

ミトコンドリアのスーパーオキシド産生量を，蛍光指示薬 MitoSOX（Invitrogen，東京）で測定した[8)]．すなわち，2 Well スライドチャンバーで内皮細胞を培養し，高濃度グルコース溶液（最終濃度 15，33 mM）またはマンニトール溶液（最終濃度 33 mM）を添加して 48 時

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[4)]熊本大学医学部附属病院　循環器臨床研究先端医療寄附講座

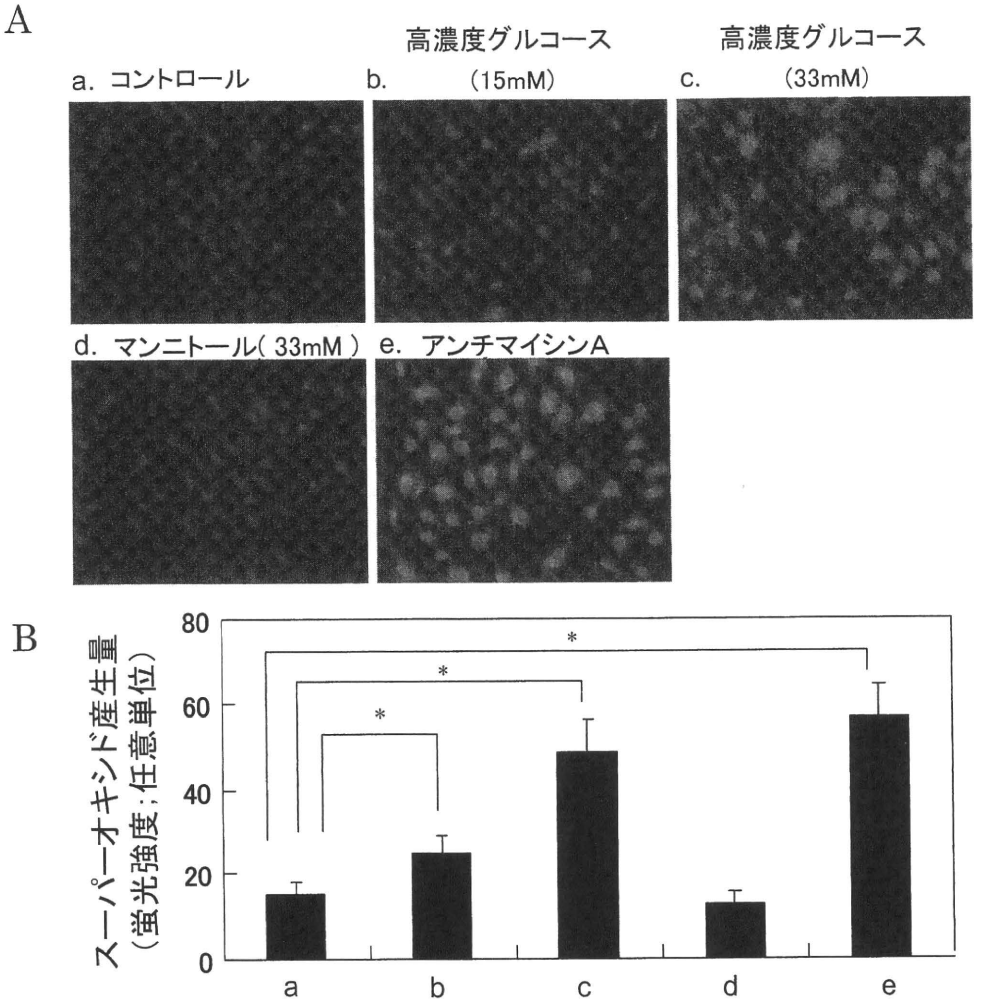

Fig. 1　高濃度グルコース処理によるミトコンドリアのスーパーオキシド産生増大
A：内皮細胞を，高濃度グルコース溶液（15 mM，33 mM），マンニトール溶液（33 mM）でそれぞれ48時間処理した．MitoSOX処理後，アンチマイシンA（100 μM）を30分間処理し，ミトコンドリアのスーパーオキシド産生量を測定した．コントロール群の培養液中には5.5 mMグルコースが含まれる．B：蛍光強度を定量化し，スーパーオキシド産生量を数値化し，平均値 ± 標準偏差（n = 5）で表した．（*$p < 0.01$；コントロール群との比較）

間培養後，MitoSOX（最終濃度5 μM）を添加し，30分間処理した．アンチマイシンA（SIGMA，東京）（最終濃度100 μM）を，MitoSOX処理した内皮細胞に添加し，30分間処理した．その後，細胞をPBSで2回洗浄し，2% パラフォルムアルデヒド（PFA）で5分間室温にて固定後，VECTASHIELD Mounting Medium with DAPI（フナコシ，東京）で封入し，蛍光顕微鏡 ECLIPSE TE 2000-S（Nikon，東京）で蛍光強度を測定した．siRNA導入細胞での実験は，導入後24時間あるいは48時間後の内皮細胞を用いて，高濃度グルコース処理した．

3）ミトコンドリア障害（膜電位変動）の測定

ミトコンドリア障害を，膜電位変動で測定した．蛍光指示薬JC-1（Cell Technology Inc，米国）は，ミトコンドリアに取り込まれる色素で，ミトコンドリアが正常の高い膜電位を持つ状態では赤色蛍光を発し，障害を受けて低い膜電位状態では緑色蛍光を発する[9]．4 Wellスライドチャンバーで内皮細胞を培養し，高濃度グルコース溶液（最終濃度33 mM）またはマンニトール溶液（最終濃度33 mM）を添加して60時間培養した．内皮細胞を，2,3-Dimethoxy-1-naphthoquinone（DMNQ）（SIGMA，東京）（最終濃度5 μM）で30分間処理した．また，内皮細胞を，活性酸素種消去剤 N-Acetyl-L-cysteine（NAC）（SIGMA，東京）（最終濃度100 mM）で30分間処理後，高濃度グルコース処理した．その後，JC-1（2 μg/ml）を添加し，15分間処理した．細胞をPBSで2回洗浄し，2% PFAで5分間室温にて固定後，VECTASHIELD

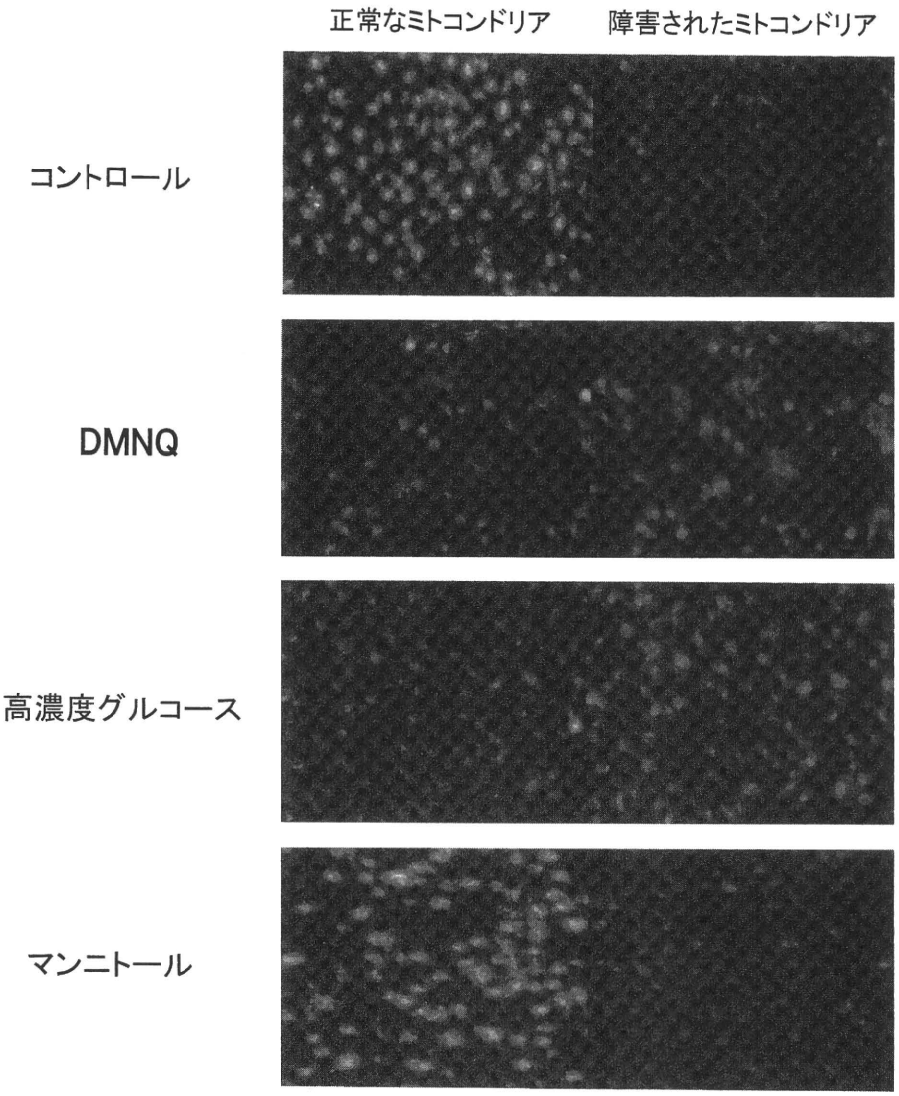

Fig. 2 高濃度グルコース処理によるミトコンドリア障害の誘導
内皮細胞を，高濃度グルコース溶液（33 mM），マンニトール溶液（33 mM），DMNQ（5 μM）でそれぞれ 60 時間処理し，蛍光指示薬 JC-1 を用いてミトコンドリア障害を測定した．赤色蛍光は正常なミトコンドリア膜電位を示し，緑色蛍光は障害されて変動したミトコンドリア膜電位を示す．

Mounting Medium with DAPI で封入し，蛍光顕微鏡 ECLIPSE TE2000-S で，蛍光強度を測定した．

4）Apop-1 発現の抑制

Apop-1 発現抑制を目的に合成された Apop-1 siRNA（QIAGEN，東京）を，Nucleofector II Device（Lonza，東京）を用い，エレクトロポレーション法により内皮細胞に導入した．スクランブル siRNA を導入した内皮細胞をコントロールとした．また，ウエスタンブロット法を用いて，Apop-1 たん白質発現の siRNA による抑制を確認した．

5）ウエスタンブロット法による Apop-1 たん白質の定量

Apop-1 と α-tublin のたん白質発現量を，それぞれ抗 Apop-1 抗体（Yasuda　O 作成，熊本）と抗 α-tublin 抗体（CALBIOCHEM，独国）を用いて，ウエスタンブロット法で定量した．すなわち，Apop-1 siRNA 導入 24 時間後あるいは 48 時間後，高濃度グルコース溶液（最終濃度 33 mM）を添加して 48 時間培養した内皮細胞を PBS で 2 回洗浄した．その後，lysis buffer（50 mM Tris-HCl pH 8.0，20 mM EDTA，1% SDS，100 mM NaCl）を加えて細胞膜を破壊し，超音波処理で均一化後，3000 rpm で 5 分間遠心分離した上清を細胞溶解液とした．

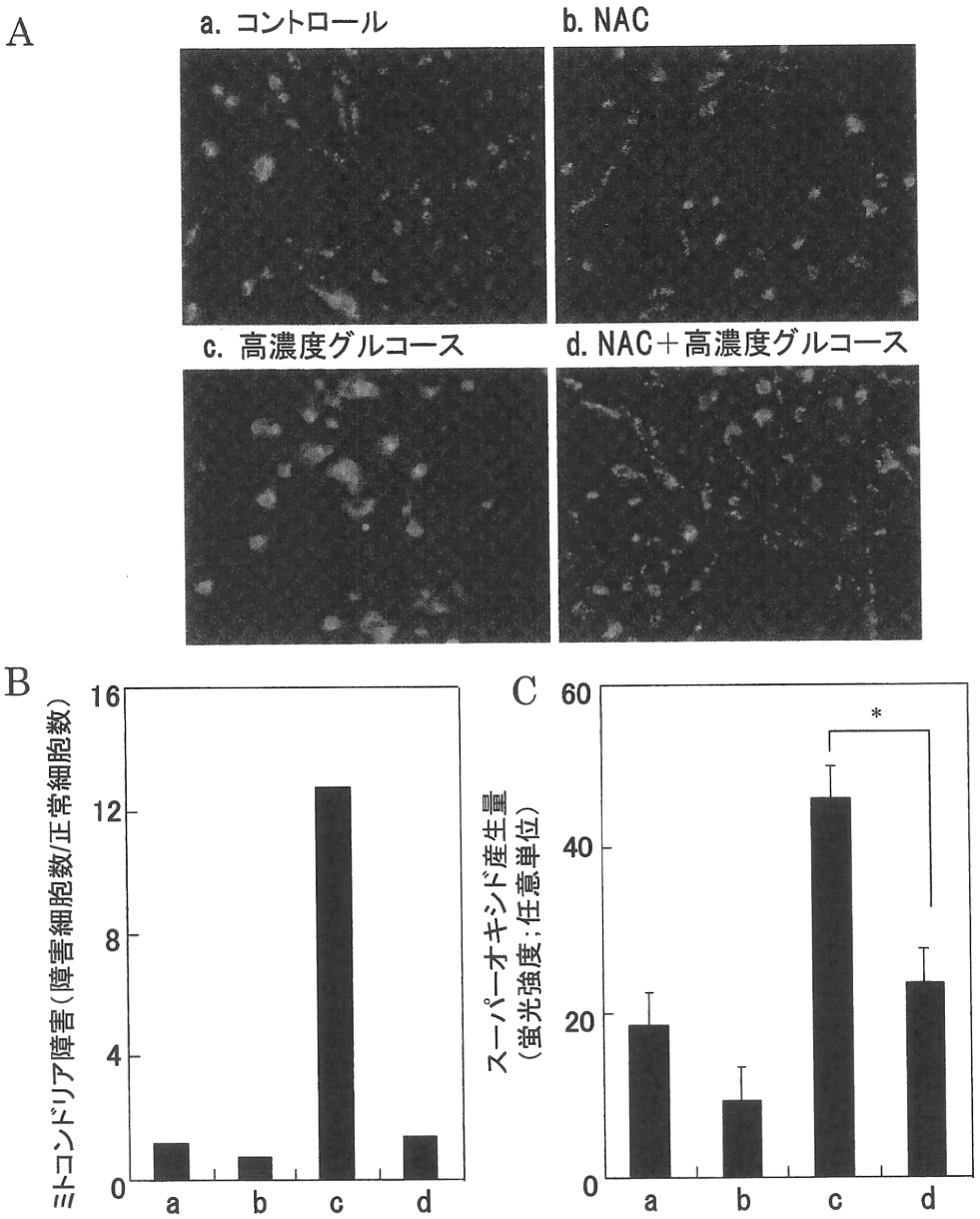

Fig. 3　活性酸素種消去剤によるミトコンドリア障害の誘導阻害

A：内皮細胞を，高濃度グルコース溶液（33 mM），あるいは高濃度グルコース溶液＋NAC（100 mM）で60時間処理し，蛍光指示薬JC-1を用いてミトコンドリア膜電位変動を測定した．JC-1の赤と緑の蛍光を重ねて示した．B:JC-1が示す赤の正常なミトコンドリアに対し，緑のミトコンドリア障害が起こった細胞の比率を定量化した．C：蛍光指示薬MitoSOXを用いてミトコンドリアのスーパーオキシド産生量を数値化し，平均値 ± 標準偏差（n = 3）で表した．（*$p < 0.01$；高濃度グルコース群との比較）

Bio-Rad Protein Assay Kit（Bio-Rad，東京）~~細胞溶解~~液でたん白質を定量後，12.5% e-PAGEL（ATTO，東京）を用いてSDS-polyacrylamide gel electrophoresis（SDS-PAGE）でたん白質を分画し，Polyvinylidene fluoride（PVDF）膜Immobilon-P（Millipore，米国）に転写した．転写膜を，3%スキムミルクを含むPBSでブロックし，一次抗体希釈液を加えて4℃で一晩振とうした．転写膜を洗浄後，HRP標識二次抗体（Wako，大阪）希釈液を加えて室温で1時間反応させた．最後に，転写膜を洗浄後，ECL Western Blotting Detection Reagents（GE Healthcare Bio-Sciences，東京）を用いた化学発光法でApop-1たん白質を検出した．画像解析には，Lumino Imaging Analyzer（Toyobo，東京）及びGel-Pro Analyzer（Media Cybernetics，米国）を用いた．

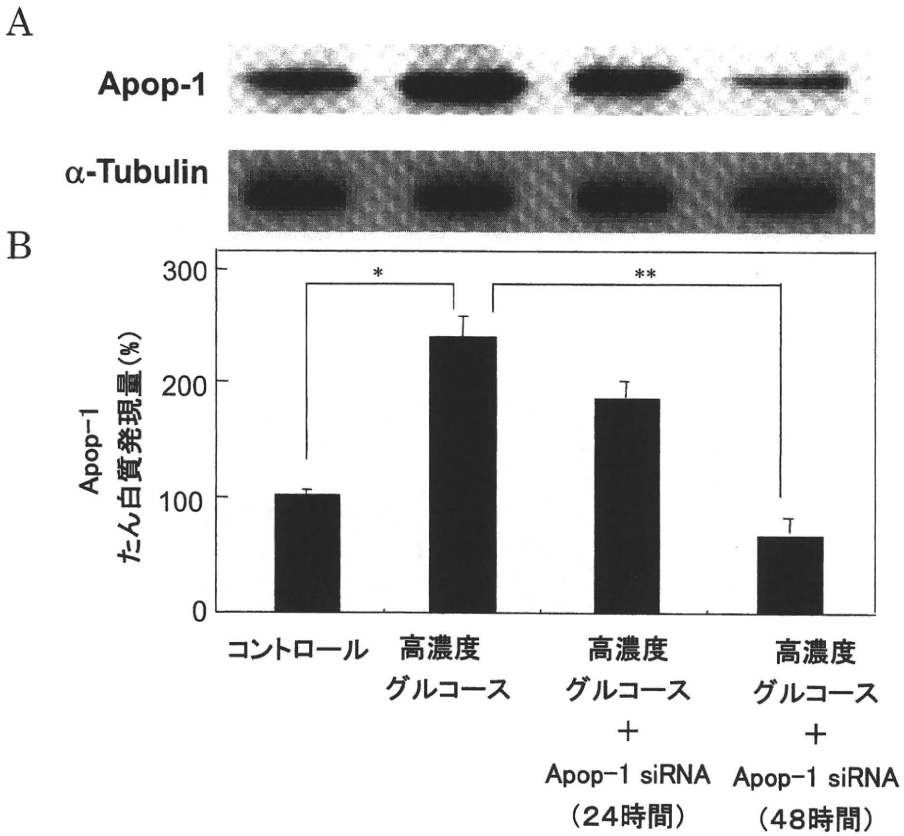

Fig. 4 高濃度グルコース処理による Apop-1 たん白質発現増大と siRNA による Apop-1 発現の抑制
A：内皮細胞を，高濃度グルコース溶液（33 mM）で 48 時間処理し，ウエスタンブロット法を用いて Apop-1 たん白質発現量を測定した．siRNA 導入 24 時間後あるいは 48 時間後に，高濃度グルコース処理した．コントロール群には，スクランブル siRNA を導入した．B：検出された Apop-1 たん白質のバンドの濃さを Image Analyzer を用いて数値化し，平均値 ± 標準偏差（n = 3）で表した．（* $p < 0.01$；コントロール群との比較，** $p < 0.01$；高濃度グルコース群との比較）α-tubulin の発現量を 1 ウエルあたり加えた試料のたん白質量が一定であることを示す内部標準とした．

6）統計解析

解析ソフト SPSS 15.0 J for Windows を用いた t 検定法により，データを統計学的に処理し，有意性を検定した．

結　果

1）高濃度グルコース処理による内皮細胞のミトコンドリアのスーパーオキシド産生増大

ミトコンドリアの活性酸素種産生を誘導するミトコンドリア complex III inhibitor アンチマイシン A[10]は，内皮細胞において，ミトコンドリアのスーパーオキシド産生を増大した（Fig. 1A，1B）．また，内皮細胞に高濃度グルコース溶液（15，33 mM）を 48 時間処理すると，5.5 mM グルコース溶液で培養したコントロール群に比し，ミトコンドリアのスーパーオキシド産生が有意に増大した．これに対し，浸透圧のみを上昇させたマンニトール溶液（33 mM）で培養した細胞では，スーパーオキシドの産生増大は認められなかった．

2）高濃度グルコース処理によるミトコンドリアのスーパーオキシド産生を介したミトコンドリア障害の誘導

次に，ミトコンドリアで産生増大したスーパーオキシドがミトコンドリア障害を誘導する可能性を検討した．内皮細胞を，高濃度グルコース溶液（最終濃度 33 mM）で 60 時間処理すると，正常なミトコンドリア膜電位を示す JC-1 の赤色蛍光強度が低下し，障害されたミトコンドリア膜電位を示す緑色蛍光強度が増大した（Fig. 2）．これに対し，マンニトール溶液（最終濃度 33 mM）

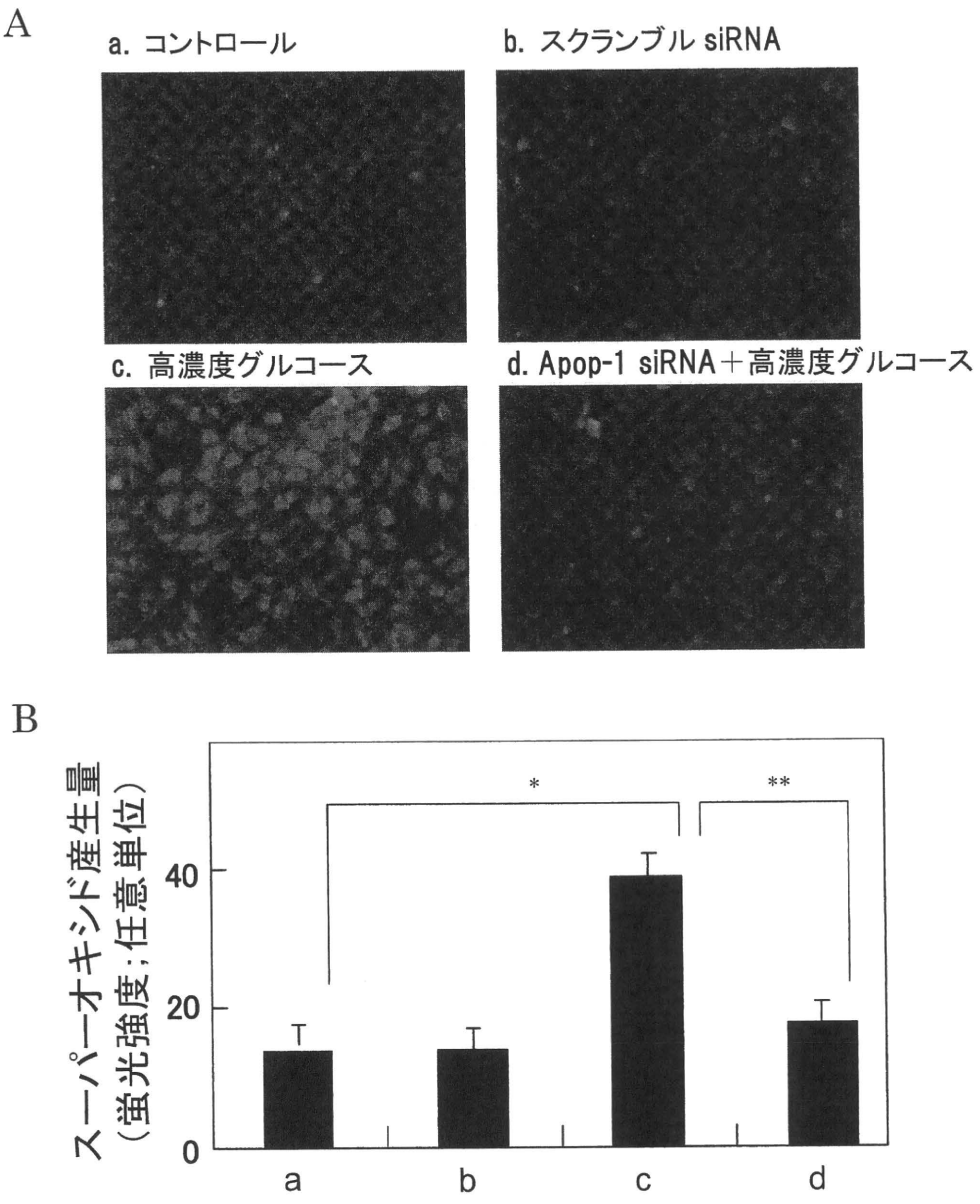

Fig. 5　Apop-1 siRNA 導入によるミトコンドリアのスーパーオキシド産生抑制
A：無処理のコントロール群（a），スクランブル siRNA 導入群（b），高濃度グルコース（33 mM）処理群（c），Apop-1 siRNA 導入 48 時間後，高濃度グルコース処理群（d），それぞれにおいて，MitoSOX を用いて，ミトコンドリアのスーパーオキシド産生量を測定した．B：蛍光強度を定量化し，スーパーオキシド産生量を数値化し，平均値 ± 標準偏差（n = 3）で表した．
（* $p < 0.01$；コントロール群との比較，** $p < 0.01$；高濃度グルコース群との比較）

で処理し，浸透圧のみを上昇させた内皮細胞では，ミトコンドリア障害は認められなかった（Fig. 2）．また，ミトコンドリアのスーパーオキシド産生を促進する DMNQ[11]は，ミトコンドリア障害を誘導した．さらに，活性酸素種消去剤 NAC の添加は，高濃度グルコース処理によるミトコンドリアのスーパーオキシド産生やミトコンドリア障害の誘導を阻害した（Fig. 3A，3B，3C）．

3）高濃度グルコース処理による Apop-1 発現増大と siRNA による Apop-1 発現抑制

次に，高濃度グルコース処理が，内皮細胞の Apop-1 発現を誘導する可能性を検討した．内皮細胞を高濃度グルコース溶液（最終濃度 33 mM）で 48 時間処理すると，コントロール群に比し，Apop-1 たん白質発現量が増大した．また，Apop-1 siRNA を導入して，48 時間後の内

皮細胞では，高濃度グルコース処理による Apop-1 たん白質発現増大が有意に抑制された（Fig. 4A，4B）．

4）高濃度グルコース処理によるミトコンドリアのスーパーオキシド産生増大に対する Apop-1 の関与

次に，高濃度グルコース処理による内皮細胞のミトコンドリアスーパーオキシド産生を Apop-1 が増強する可能性を検討した．

Apop-1 siRNA を導入して 48 時間後の，Apop-1 発現抑制した内皮細胞では，高濃度グルコース処理（最終濃度 33 mM）によるミトコンドリアのスーパーオキシド産生が有意に抑制された（Fig. 5A，5B）．

考　察

本研究は，ミトコンドリア局在たん白質 Apop-1 が，グルコースによる内皮細胞のミトコンドリアの活性酸素種産生増大の増強作用をもつことを初めて示唆したものである．

糖尿病において内皮細胞内に増大する活性酸素種は，NO の防御作用を上回るとともに，NO と反応して強力な細胞障害因子であるパーオキシナイトライト（$ONOO^-$）を産生し，細胞傷害や細胞死を誘導する[12]．内皮細胞の傷害や細胞死は，「傷害反応説」に示されているように，動脈硬化進行を促進する[13]．また，活性酸素種による内皮細胞の傷害は，細小血管障害である腎症や網膜症，神経障害など糖尿病の合併症を発症させる．

ミトコンドリア活性酸素種の産生過程には，スーパーオキシド産生系である Complex I や Complex III と，その消去系である MnSOD のバランスが関係している可能性がある．最近，ミトコンドリア活性酸素種の産生にミトコンドリア局在タンパク質 monoamine oxidase（MAO）や $p66^{Shc}$ が関与すると報告された．すなわち，MAO 作用を阻害する薬剤が活性酸素種の産生や虚血性心筋障害を抑制すること[14]，また，$p66^{Shc}$ 欠損マウスでは，ミトコンドリア活性酸素種の産生が低下し，高脂質食による動脈硬化病変が軽減した[15]．特に，$p66^{Shc}$ は，Apop-1 と同様にアポトーシス誘導作用を有するミトコンドリア局在たん白質であるため，両者がどのように関連するかを検討する必要がある．また，心筋細胞においては，ミトコンドリアの活性酸素種や細胞膜の NAD(P)H オキシダーゼを介して，初期に産生された活性酸素種が，その後のミトコンドリア活性酸素種の産生をさらに増大するポジティブフィードバック（活性酸素種による活性酸素種産生誘導）機構が存在する[16]．内皮細胞にも，このような機構が存在するか検討する必要がある．

一方，ミトコンドリアによる活性酸素種の産生増大は，ミトコンドリア機能障害を誘導する．例えば，ミトコンドリアの酵素である aconitase，α-ketoglutarate dehydrogenase，pyruvate dehydrogenase，および complexes I，II，III などが活性酸素種に強い感受性を持ち，酵素活性低下をおこす[17]．また，adenine nucleotide translocase（ANT）は，活性酸素種により酵素活性を低下させるため，細胞の ATP 産生を低下させると報告されている[18]．このような，内皮細胞のミトコンドリア機能障害は，内皮機能低下やアポトーシスを誘導し，動脈硬化の発症を促進する可能性がある[19]．

以上，本研究は，グルコースによる内皮細胞のミトコンドリア活性酸素種の産生をミトコンドリア局在たん白質 Apop-1 が増強することを示唆したものであり，この結果は，今後，糖尿病による動脈硬化の進行に対する Apop-1 をターゲットにした新しい治療法の開発に貢献する可能性を示している．

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Apop-1, a Novel Mitochondrial Protein, May Enhance the Production of Mitochondrial Reactive Oxygen Species by Glucose Treatment in Endothelial cells

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Accumulating evidence shows that mitochondria-derived reactive oxygen species (ROS) play an important role in the genesis of diabetes mellitus as well as apoptosis and ageing. However, the molecular mechanism of ROS production from the mitochondria remains unclear. Here, we examined whether Apop-1, a novel mitochondrial protein, participates in the induction of mitochondria-derived ROS production by glucose treatment in endothelial cells. Glucose treatment induced an increase in the levels of mitochondria-derived ROS and mitochondrial dysfunction in these cells. N-acetylcysteine (NAC), however, inhibited glucose-induced mitochondrial dysfunction. Apop-1 protein expression was up-regulated by glucose treatment and siRNA-mediated knockdown of the expression of Apop-1 protein significantly reduced glucose-treated mitochondria-derived ROS levels in endothelial cells. These results suggest that Apop-1 may be involved in the pathogenesis of diabetes mellitus by up-regulating ROS production from the mitochondria in endothelial cells.

Key words: Apop-1, mitochondria, reactive oxygen species, endothelium

Survival Analysis of Patients With Duodenal Gastrointestinal Stromal Tumors

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Goals: To evaluate the survival characteristics of patients with duodenal gastrointestinal stromal tumors (GISTs).

Background: GISTs represent the most common mesenchymal neoplasms. However, duodenal GISTs are relatively rare, and few studies have been performed with a focus on duodenal GISTs.

Study: We collected the data of 41 GIST patients including 7 duodenal cases. Clinicopathologic findings and recurrence-free survival (RFS) of duodenal GIST patients were analyzed.

Results: The proportion of having any symptoms was 86% in duodenum, 32% in stomach, and 56% in other GISTs ($P = 0.034$), and the most common symptoms of duodenal GISTs were melena and anemia. The 2-year RFS rates were 51.4% in duodenal GISTs, 78.4% in stomach GISTs, and 100% in other GISTs, and duodenal GISTs showed poorer RFS than nonduodenal GISTs (hazard ratio, 5.1; log-rank $P = 0.019$). Particularly, in low-risk and intermediate-risk group, the hazard ratio of recurrence was 12.3 (log-rank $P = 0.010$). Multivariate Cox analysis showed symptom ($P = 0.007$), mitotic index ($P = 0.011$), and tumor location ($P = 0.043$) were significant prognostic factors of recurrence.

Conclusions: RFS of duodenal GISTs was worse than nonduodenal GISTs.

Key Words: duodenum, GIST, RFS, survival

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Gastrointestinal stromal tumors (GISTs) represent the most common mesenchymal neoplasms arising within the gastrointestinal tract. These tumors are thought to share a common progenitor cell with the interstitial cells of Cajal, and usually have activating mutations in either c-kit (75% to 80%) or platelet-derived growth factor receptor α (PDGFRA) (5% to 10%), 2 closely related receptor tyrosine kinases.[1] These mutations lead to ligand-independent activation and signal transduction mediated by constitutively activated KIT or PDGFRA. This theory was first proposed by Kindblom et al[2] and Hirota et al[3] revealed an association between the presence of c-kit mutation and tumor development.

GISTs can arise anywhere in the gastrointestinal tract, but their most frequent locations are the stomach (60%) and the small intestine (25%). Duodenal GISTs are relatively rare and comprise about 5% of surgically resected GIST cases.[4,5] Earlier studies have reported that duodenal GISTs were larger than stomach GISTs, and that their most frequent locations were the second and third portions of the duodenum.[4] Owing to the unique and complex anatomy of the duodenum, complete resection of duodenal GISTs sometimes requires wide resection methods such as pancreaticoduodenectomy,[6] which is rarely the case for GISTs in other locations. Only few reports about the characteristics of duodenal GISTs have been published earlier,[7–9] and few studies have been performed a survival analysis of patients with duodenal GISTs. From August 1993 to January 2008, we encountered 41 GIST cases of which 7 were duodenal GISTs. Here we conduct a retrospective cohort study to evaluate the survival characteristics of duodenal GISTs.

PATIENTS AND METHODS

Patients

We retrospectively reviewed the records of all patients with GISTs treated at the Osaka National Hospital between August 1993 and January 2008. The diagnosis of GISTs was conducted by histologic examination, immunohistochemical staining for KIT and CD34, and detection of c-kit or PDGFRA mutations.

Data on patients' age, sex, tumor location, symptoms, pathologic findings, c-kit and PDGFRA mutations, treatment, and survival outcome were collected. Tumor size was defined as the largest diameter of the primary tumor in any dimension. Pathologic data included mitotic index and results of immunohistochemical staining for KIT and CD34. Treatment data included type of resection and adjuvant treatment. Tumor size and mitotic index were used for risk classification according to the Fletcher score.[10] However, in this study, we combined low-risk and intermediate-risk patients in the survival analysis because "low-risk" has not been defined for duodenal GISTs.

Statistical Analysis

Associations between tumor location and clinicopathologic variables were analyzed using the χ^2 test. Recurrence-free survival (RFS) was defined as the time

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TABLE 1. Characteristics of 7 Patients With Duodenal GISTs

Age (y)	Sex	Clinical Symptom	Location of Duodenal GIST	Size (mm)	Operation	Adjuvant Therapy	KIT	CD34	Mitotic Index (per 50 HPF)	*C-Kit* Mutation	Risk Classification
64	F	Melena	Second part	60	Gastrojejunostomy	–	–	–	< 5	Exon 11	Intermediate
58	F	Melena	Second part	70	Pancreaticoduodenectomy	–	+	+	< 5	NA	Intermediate
70	F	Abdominal mass	Fourth part	150	Partial duodenal resection	+	+	+	< 5	Exon 13	High
67	F	Absent	First part	60	Partial duodenal resection	–	+	+	< 5	Exon 11	Intermediate
39	F	Melena	First part	120	Partial duodenal resection	–	+	+	5-10	Exon 11	High
65	M	Anemia	Second part	30	Partial duodenal resection	–	+	NA	5-10	Exon 9	Intermediate
75	F	Anemia	Second part	40	Pancreaticoduodenectomy	–	+	–	> 10	Exon 11	High

GISTs indicates gastrointestinal stromal tumors; HPF, high-power field; F, female; M, male; NA, not analyzed.

from surgery to either the first recurrence or death from any cause. RFS curves were estimated by the Kaplan-Meier method and compared using the log-rank test. Multivariate Cox regression analyses were performed to adjust for the potential confounding factors whose *P* values were under 0.2 in univariate analyses. All statistical analyses were performed with SPSS software, version 15.0J. *P* values less than 0.05 were considered statistically significant, and all tests were 2-sided.

RESULTS

Patient Characteristics

Forty-one patients with GISTs were admitted for treatment to Osaka National Hospital between August 1993 and January 2008, and of these 7 patients (17%) were diagnosed with duodenal GISTs (Table 1). Six of the 7 duodenal GIST patients were female. The second portion of the duodenum was most frequently affected, which is of significance because of the need for pancreaticoduodenectomy if the tumor is located on the same side of intestine as the Papilla Vater. For 1 duodenal GIST patient, we could not perform radical surgery because of severe patient's general condition, whereas the other duodenal GIST patients received complete gross resection. Postoperative complications occurred in 3 of 7 duodenal GIST patients. These complications included pancreatic fistula, and intra-abdominal abscess, but none of the patients died within 1 month after surgery. Only 1 patient received adjuvant chemotherapy after surgery. One patient showed immunohistochemical staining of neither c-kit nor CD34. Six cases had c-kit mutations; 4 for exon 11, 1 for exon 9, and 1 for exon 13. The numbers of intermediate-risk and high-risk patients were 4 (57%) and 3 (43%), respectively.

We compared patients with duodenal GISTs to those with stomach GISTs and other GISTs (Table 2). Among 9 patients with other GISTs, 4 were found in rectum and in small intestine, and 1 in omentum. There were no statistical differences in clinicopathologic factors except for clinical symptoms and CD34 positivity. With regard to immunohistochemical findings, the KIT-positive rate was similar in duodenal and other GISTs, whereas the CD34-positive rate was lower in duodenal GISTs ($P = 0.049$). Although over 30% patients with stomach GISTs were classified as low-risk, there were no low-risk patients among the duodenal and other GIST groups.

Of patients with duodenal GISTs, 86% had symptoms, whereas 32% of patients with stomach GISTs, and 56% of those with other GISTs were affected; this difference was statistically significant ($P = 0.034$). Five of 6 symptomatic patients with duodenal GISTs had melena or anemia, whereas a half of symptomatic patients with stomach GISTs complained of epigastralgia (Table 3).

TABLE 2. Comparison of Characteristics Among Duodenal GISTs, Stomach GISTs, and GISTs in Other Locations

	Duodenum (n = 7)	Stomach (n = 25)	Other (n = 9)	*P*
Age (y)				0.50
Median (range)	65 (39-75)	67 (48-82)	59 (45-86)	
Sex				0.10
Male	1 (14%)	13 (52%)	2 (22%)	
Female	6 (86%)	12 (48%)	7 (78%)	
Clinical Symptom				0.034
Absent	1 (14%)	17 (68%)	4 (44%)	
Present	6 (86%)	8 (32%)	5 (56%)	
Immunohistochemistry				
KIT				0.53
Positive	6 (86%)	22 (88%)	9 (100%)	
Negative	1 (14%)	3 (12%)	0 (0%)	
CD34*				0.049
Positive	4 (67%)	23 (96%)	6 (67%)	
Negative	2 (33%)	1 (4%)	3 (33%)	
Tumor size (cm)†				0.40
Median (range)	6.0 (3.0-15)	5.0 (1.7-24)	6.0 (2.5-12)	
Mitotic Index (per 50 HPF)				0.59
< 5	4 (57%)	16 (64%)	3 (33%)	
5-10	2 (29%)	5 (20%)	3 (33%)	
> 10	1 (14%)	4 (16%)	3 (33%)	
Risk Classification				0.13
Low	0 (0%)	8 (32%)	0 (0%)	
Intermediate	4 (57%)	7 (28%)	5 (56%)	
High	3 (43%)	10 (40%)	4 (44%)	
C-kit mutation‡				0.33
Exon 9	1 (17%)	0 (0%)	1 (50%)	
Exon 11	4 (66%)	12 (86%)	1 (50%)	
Exon 13	1 (17%)	1 (7%)	0 (0%)	
Exon 17	0 (0%)	1 (7%)	0 (0%)	

*One duodenal GIST case and 1 stomach GIST case were not analyzed.
†One stomach GIST case was not analyzed.
‡One duodenal GIST case, 11 stomach GIST cases, and 7 other GIST cases were not analyzed.
GISTs indicates gastrointestinal stromal tumors; HPF, high-power field.

TABLE 3. Symptoms Among Duodenal GISTs, Stomach GISTs, and GISTs in Other Locations

	Duodenum (n = 6)	Stomach (n = 8)	Other (n = 5)
Anemia	2 (33%)	2 (25%)	0
Melena	3 (50%)	0	1 (20%)
Epigastralgia	0	4 (50%)	0
Abdominal mass	1 (17%)	1 (13%)	1 (20%)
Nausea	0	1 (13%)	1 (20%)
Others	0	0	2 (40%)

GISTs indicates gastrointestinal stromal tumors.

Survival

In survival analysis of all GIST patients, the 2-year RFS rates of duodenal, stomach, and other GISTs were 51.4%, 78.4%, and 100%, respectively ($P = 0.058$) (Fig. 1A). As the survival curves of stomach and other GISTs were similar, we combined the stomach GISTs with other GISTs as a nonduodenal group, and compared RFS of duodenal GIST patients with those of nonduodenal GIST patients. As the result, the hazard ratio (HR) of recurrence was 5.1 [95% confidence interval (CI), 1.1-23.2] in the duodenal GIST patients, and the log-rank test showed statistical significance ($P = 0.019$) (Fig. 1B). In the low-risk and intermediate-risk groups specifically, the 2-year RFS rates of patients with duodenal and nonduodenal GISTs were 50% and 100%, respectively, showing a statistical difference (log-rank $P = 0.010$) and the HR of recurrence was 12.3 (95% CI, 1.1-142.9) (Fig. 2A). However, in the high-risk group there was no significant difference in RFS between duodenal GIST patients and nonduodenal GIST patients (log-rank $P = 0.60$) (Fig. 2B), and the HR of recurrence was 1.8 (95% CI, 0.19-17.9).

Univariate analyses revealed that symptom ($P = 0.009$), mitotic index ($P = 0.038$), and tumor location ($P = 0.035$) were the statistically significant prognostic factors of RFS (Table 4). These 3 factors were significantly associated with RFS even in multivariate analysis.

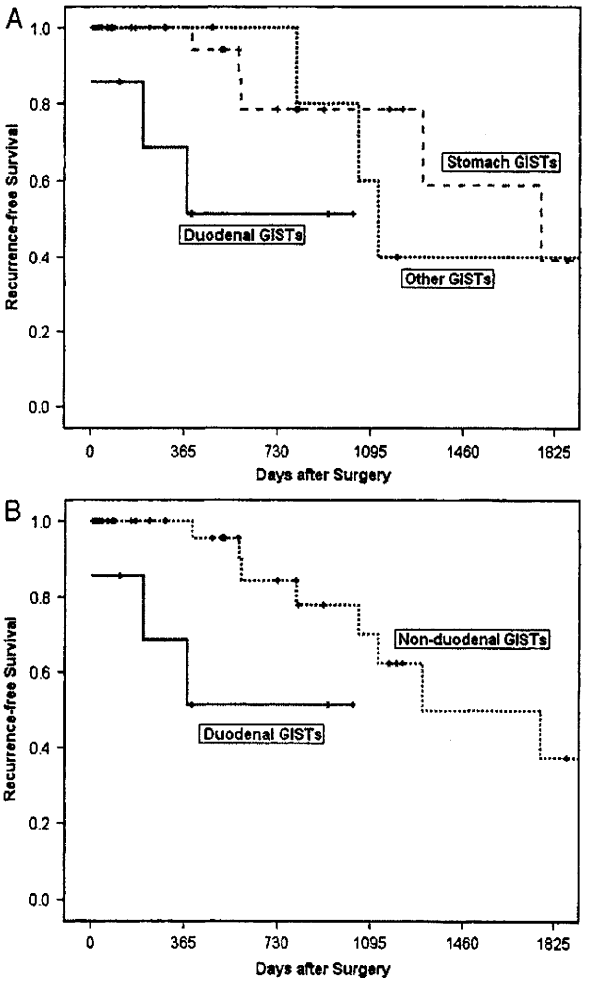

FIGURE 1. Recurrence-free survival of patients with gastrointestinal stromal tumors (GISTs) on the basis of tumor location. A, Duodenal versus stomach versus other GISTs. B, Duodenal versus nonduodenal GISTs.

DISCUSSION

GISTs are often discovered in the stomach and small intestine, but duodenal GISTs comprise only about 5% of these. Although 2 case series have studied duodenal GISTs,[7,8] neither conducted a survival analysis. This study showed that the RFS of duodenal GIST patients was worse than that of patients with stomach GISTs or GISTs in other locations, and the poor prognosis of duodenal GISTs was more remarkable in low-risk and intermediate-risk patient groups. Several earlier studies have reported that patients with GISTs of the small intestine have an unfavorable prognosis, compared with stomach GISTs.[11-13] In this study, we combined small intestine cases with stomach cases, because the survival curves of stomach and other GISTs were similar. Multivariate Cox analyses performed after adjusting for other prognostic factors revealed that tumor location was

TABLE 4. Association of Clinicopathological Factors With Recurrence-free Survival

	Univariate		Multivariate	
	Hazard Ratio (95% CI)	P	Hazard Ratio (95% CI)	P
Age (>65 y)	2.4 (0.64-9.2)	0.20	1.6 (0.34-7.3)	0.56
Sex (male)	1.4 (0.43-4.8)	0.55	—	—
Symptom (present)	17.4 (2.0-150.1)	0.009	158.1 (3.9-6374.0)	0.007
Tumor size (>5 cm)	1.6 (0.45-5.7)	0.47	—	—
Mitotic index (≥5/50 HPF)	5.1 (1.1-23.8)	0.038	37.0 (2.3-596.7)	0.011
Location (duodenum)	5.1 (1.1-23.3)	0.035	10.9 (1.1-111.1)	0.043

CI indicates confidence interval; HPF, high-power field.

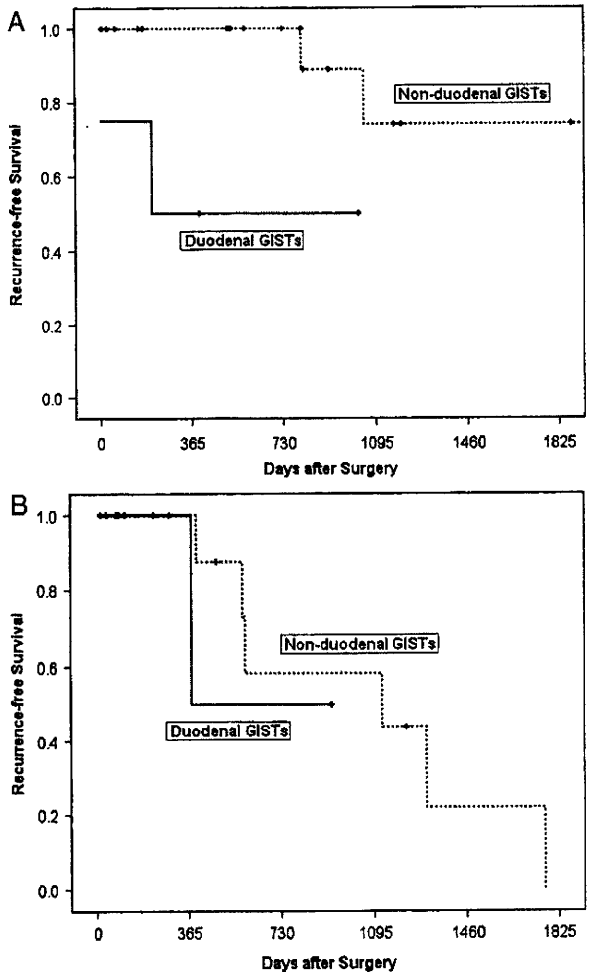

FIGURE 2. Recurrence-free survival of patients with duodenal gastrointestinal stromal tumors (GISTs) and nonduodenal GISTs in (A) low-risk and intermediate-risk group and (B) high-risk group.

an independent prognostic factor for GISTs. This result may indicate that the duodenal GISTs are biologically different from other GISTs.

Over the past several years, site-specific differences in appearance, morphology, and clinical outcome have been identified in GISTs. It has been reported that the proportion of CD34-positive tumors and the frequency of c-kit mutations are different depending on location.[14,15] An earlier study reported that CD34 positivity was more frequent in malignant tumors than in borderline or benign tumors.[16] Another study reported that CD34 positivity in patients with recurrence is higher than those without recurrence, although the difference was not statistically significant.[17] In this study, the proportion of CD34-positive patients with duodenal GISTs was even lower than that in patients with stomach GISTs. Thus, we cannot explain the poor survival of patients with duodenal GISTs by CD34 positivity alone. In contrast, earlier studies showed that mutations of exon 9 were more common in patients with small intestinal GISTs than in those with stomach GISTs.[18,19] GISTs with exon 9 mutations are often clinically and pathologically malignant, and this subgroup of patients is often resistant to imatinib. In our population, a duodenal GIST patient with an exon 9 mutation showed early metastases to the liver after surgery. The positivity rate of c-kit exon 9 mutations may contribute to the poor survival of patients with duodenal GISTs. In this study, however, we did not analyze c-kit mutation sites for about half of all GIST cases, so we could not evaluate the association between survival and the location of c-kit mutation.

In comparison of clinicopathologic characteristics among 3 location types of GISTs, clinical symptom was the most significant finding. Many duodenal GIST patients had symptomatic complaints that were mainly associated with bleeding from tumor, whereas the proportion of stomach GIST patients who had any clinical symptoms in diagnosis was low (28%). Most of asymptomatic patients with stomach GISTs were diagnosed in medical screening or follow-up of other diseases. In Japan, medical screening with upper gastrointestinal endoscopy or x-ray has been widespread because of high prevalence of gastric cancer, and it may contribute to early detection of asymptomatic stomach GISTs. These features may induce the survival difference between the duodenal and nonduodenal GISTs. However, the tumor location was an independent prognostic factor after adjusting for the presence of clinical symptoms in the multivariate Cox analyses.

Surgery remains the mainstay of treatment for patients with primary GISTs without distant metastasis. A recent retrospective study to compare the survivals of duodenal GIST patients after pancreaticoduodenectomy with those after limited resection reported that the disease-free survivals were similar between 2 surgical procedures.[6] In this study, both the 2 cases who received pancreaticoduodenectomy are alive without recurrence, whereas 2 of 4 patients who received limited duodenal resection had recurrence after surgery. Complete gross resection with an intact pseudocapsule may be the most important thing to treat duodenal GISTs, and so we should not hesitate to perform combined resection such as pancreaticoduodenectomy to achieve gross resection, even though the surgical procedure is highly invasive.

Limitations of this study include its retrospective design and small sample size. As survival analyses with small number of patients sometimes mislead the results, we should therefore be careful in evaluating its results. However, to our knowledge, this is the first study to focus on the survival of patients with duodenal GISTs, and the difference of RFS between duodenal and nonduodenal GISTs was remarkable. In the future, prospective studies using larger numbers of patients will be needed.

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Influence of Bursectomy on Operative Morbidity and Mortality After Radical Gastrectomy for Gastric Cancer: Results of a Randomized Controlled Trial

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Abstract

Background Bursectomy, a procedure dissecting the peritoneal lining covering the pancreas and the anterior plane of the transverse mesocolon, has been commonly performed with radical gastrectomy for gastric cancer patients. Although possibly improving the prognosis of gastric cancers, adverse events related to bursectomy should be evaluated in prospective studies.

Methods This prospective randomized controlled trial was conducted by experienced surgeons in 11 Japanese institutions. Patients with T2 or T3 gastric adenocarcinoma were intraoperatively randomized to radical gastrectomy plus D2 lymphadenectomy either with or without bursectomy. Postoperative morbidity and mortality were compared between the two groups.

Results A total of 210 patients were assigned to the bursectomy group (104 patients) and the nonbursectomy group (106 patients) between July 2002 and January 2007. Background characteristics were well balanced. Intraoperative blood loss was greater in the bursectomy group than in the nonbursectomy group (median 475 vs. 350 ml, $p = 0.047$), whereas other surgical factors did not vary significantly. The overall morbidity rate was 14.3% (30 patients), the same for the two groups. Likewise, the incidence of major postoperative complications, including pancreatic fistula, anastomotic leakage, abdominal abscess, bowel obstruction, hemorrhage, and pneumonia, were not significantly different between the two groups. The medians of the amylase level of the drainage fluid on postoperative day 1 were similar for the two groups (median 282 vs. 314 IU/L, $p = 0.543$). The hospital mortality rate was 0.95%: one patient per group.

Conclusions Experienced surgeons could safely perform a D2 gastrectomy with an additional bursectomy without increased major surgical complications.

Introduction

More than half of the new cases of gastric cancer occur in eastern Asia [1]. The surgical intervention for gastric cancers has rapidly developed in Japan. An extended radical lymphadenectomy, which is almost identical to the present D2 dissection, along with bursectomy was established as the standard treatment for advanced gastric cancers during the early 1960s [2, 3]. Bursectomy is a traditional surgical procedure to dissect the peritoneal lining covering the pancreas and the anterior plane of the transverse mesocolon with an omentectomy [4, 5]. This procedure is recommended in the Japanese Gastric Cancer Treatment Guidelines as part of the radical surgery for gastric cancer to remove micrometastases disseminated into the bursa omentalis [6]. As gastric cancer in the posterior wall sometimes shows peritoneal dissemination only in the bursa omentalis, its resection may improve survival [7].

On the other hand, a bursectomy causes some surgical stress when performed in addition to a D2 lymph node

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dissection. Therefore, the possible increase in the incidence of postoperative complications, including pancreatic fistula formation, intestinal obstruction, and hemorrhage, may be concerning. As the safety of a D2 lymph node dissection is still controversial in Western countries [8, 9], we should also carefully evaluate the safety of bursectomy. To elucidate the safety and usefulness of the bursectomy, we conducted a multiinstitutional randomized controlled trial. We hereby present our operative morbidity and mortality data, the secondary endpoints of this trial. The final analysis of survival data is scheduled to take place in 2012.

Patients and methods

Patients

Patient eligibility criteria for this study were as follows: (1) histologically proven primary adenocarcinoma of the stomach; (2) a preoperative and intraoperative classification of T2N0, T3N0, T2N1, or T3N1 according to 13th edition of the Japanese Classification of Gastric Carcinoma [10]; (3) a lack of noncurative surgical factors except for positive lavage cytology; (4) no Borrmann type 4 (linitis plastica) cases; (5) no prior chemotherapy or radiation therapy; (6) ages 20 to 80 years with a performance status of 0 to 2 according to the Eastern Cooperative Oncology Group (ECOG) scale; (7) no history of gastrectomy or other malignancy during the last 5 years. All patients gave written informed consent before undergoing randomization.

When the surgeon confirmed the above eligibility criteria immediately after the initial laparotomy, patients were then intraoperatively randomized to the bursectomy group (a D2 gastrectomy with bursectomy) or the nonbursectomy group (without bursectomy). Randomizations were made by the minimization method according to sex, clinical T stage (cT2 vs. cT3), and gastrectomy (total vs. distal subtotal gastrectomy).

Surgery

In both the bursectomy and nonbursectomy groups, the surgeon performed a total or distal subtotal gastrectomy and D2 lymph node dissection as a standard treatment for advanced gastric cancers [10]. With total gastrectomy for T2 or deeper tumors in the proximal third of the stomach, the spleen was removed in principle for splenic hilar lymphadenectomy. Pancreatectomy was confined to those patients whose pancreas was involved by tumor.

An omentectomy was performed for both groups in this study. In the bursectomy group, the peritoneal lining of the bursa omentalis was removed en bloc as much as possible from the anterior plane of the transverse mesocolon and the pancreas. In the caudal area of the bursa omentalis, the anterior lesion was removed with the minor omentum at the edge of the left lobe of the liver. The posterior and right-sided lesions were removed with lymph node dissection along the common hepatic artery (no. 8a), the splenic artery (no. 11p/d), the left gastric artery (no. 7), and in the hepatoduodenal ligament (no. 12a). As complete removal of the left side of the bursa omentalis did not allow a distal subtotal gastrectomy, pancreatic serosa was removed up to the proximal half of the splenic artery (no. 11p). For the transverse colon mesentery, the peritoneum was removed up to the left gastroepiploic artery (no. 4sb). In the nonbursectomy group, the right anterior surface of the transverse colon mesentery was partially removed around the root of the right gastroepiploic artery (no. 6). Only a small amount of peritoneum could be removed for lymph node dissection. Thus, the bursa omentalis peritoneal lining was preserved as much as possible in the nonbursectomy group. The type of reconstruction and the indication of prophylactic cholecystectomy were not specified in the protocol.

Patients were enrolled from 11 hospitals belonging to the Osaka University Clinical Research Group for Gastroenterological Surgery. More than 50 gastrectomies were performed each year in these 11 hospitals. All operations were performed or supervised by senior surgeons who were members of the Japanese Gastric Cancer Association. During the planning of the study, all participating surgeons reached an agreement concerning the technical details of bursectomy.

Postoperative evaluation

Operative methods and pathology results were recorded according to the 13th edition of the Japanese Classification of Gastric Carcinoma [10]. The number of dissected lymph nodes was measured by pathology. Drainage fluid was collected via an operatively placed drain on postoperative day (POD) 1 for measuring the amylase level. The six Representative data for the six major morbidities—pancreatic fistula, anastomotic leakage, abdominal abscess, bowel obstruction, hemorrhage, pneumonia—were prospectively collected. A pancreatic fistula was defined by a drainage output on or after POD 5 with an amylase content more than three times the upper normal serum value. Pneumonia, anastomotic leakage, abdominal abscess, and bowel obstruction were diagnosed radiologically or clinically. Postoperative hemorrhage requiring a transfusion was recorded as morbidity. Any other complications requiring pharmacologic or surgical treatment were recorded on a free format. Operative morbidity until 3 months after surgery was also analyzed in this study. Operating time, blood loss, duration of hospital stay after surgery, and reoperation details were also recorded. Hospital mortality

was defined as postoperative death of any cause within 30 days or death during the same hospitalization.

Patients were followed every 3 months until 5 years after the operation. Adjuvant therapy was not permitted before a recurrence of cancer.

Statistical Analysis

The primary endpoint was overall survival (OS). Secondary endpoints were recurrence-free survival, operative morbidity, and POD 1 drainage amylase levels. We planned initially to recruit 200 patients, with an alpha error of 0.1 and statistical power of 80%. This allowed detection of a 10% margin of noninferiority for the nonbursectomy group under the estimation of a 60% 5-year OS in the bursectomy group. The projected accrual period and follow-up period were 3 years and 5 years, respectively. After registration of 204 patients, we amended the sample size and analysis to correct the estimation of the 5-year OS in the bursectomy group as 75% and to reduce alpha error. The amended sample size was 464, with an alpha error of 0.05 and statistical power of 80%, with an 8-year accrual period (total) and 5-year follow-up.

In January 2007, the positive result of a large-scale randomized controlled trial to evaluate adjuvant S-1 chemotherapy for stage II/III gastric cancer patients was reported [11, 12]. Since then, adjuvant S-1 chemotherapy has been a new standard treatment for stage II/III gastric cancer patients in Japan. However, because any adjuvant treatment including S-1 was not allowed after surgery in our study, we decided to close the accrual of our study in January 2007.

The operative morbidity and mortality rates were based on the proportion of the number of cases divided by all registered patients based on the intention-to-treat principle. The differences in proportion between the two groups were evaluated using Fisher's exact test or chi-squared test. The differences of continuous variables, including age, body mass index, tumor size, operating time, blood loss, and the number of dissected lymph nodes for the two groups were tested with a Mann-Whitney U-test. All p values were two-sided, and statistical analysis was done using SPSS Statistics software, version 17.0 (SPSS, Chicago, IL, USA).

Results

Patients and surgery

Between July 2002 and January 2007, a total of 210 patients were randomly divided into 104 in the bursectomy group and 106 in the nonbursectomy group (Fig. 1). One patient in the bursectomy group did not undergo bursectomy, and one in the nonbursectomy group underwent

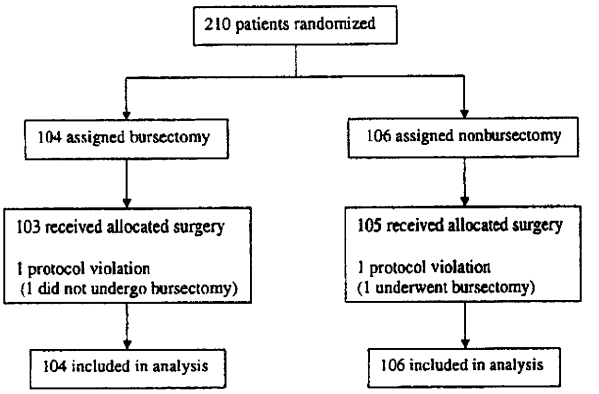

Fig. 1 CONSORT flowchart for patients

bursectomy. Most of the baseline characteristics were well balanced (Table 1). The bursectomy group had slightly older patients than the nonbursectomy group (median 65 vs. 63 years, $p = 0.099$). The number of patients with pathologically positive nodes was slightly higher in the bursectomy group than in the nonbursectomy group (52.9% vs. 43.4%, $p = 0.214$).

The operative details are shown in Table 2. A total gastrectomy was performed on 22 (21.2%) patients in the bursectomy group and on 27 (25.5%) patients in the nonbursectomy group. About one-half of patients in each of the two groups underwent a Roux-en-Y reconstruction procedure. A combined resection of other organs was performed for 103 patients in total. The resected organs were the gallbladder in 98 patients, spleen in 26 patients, part of the pancreas in 1 patient, the colon in 1 patient, the left adrenal gland in 1 patient, and the diaphragm in 1 patient. It was of note that although the difference was not statistically significant the number of patients with a combined resection was greater in the nonbursectomy group than in the bursectomy group (42.3 vs. 55.7%, $p = 0.055$). When we evaluated the operating time after dividing the patients into two subgroups, either with or without a combined resection of other organs, the bursectomy required a longer operating time (median 27 min in patients with a combined resection, 26 min in patients without a combined resection). The amount of blood loss significantly increased in the bursectomy group compared to the nonbursectomy group (median 475 vs. 350 ml, $p = 0.047$). There was no significant difference between the two groups regarding the number of dissected lymph nodes.

Operative morbidity and mortality

The overall operative morbidity rate was 14.3% (30 patients), which was the same in the two groups (Table 3). Prespecified complications, including pancreatic fistula, anastomotic leakage, abdominal abscess, bowel obstruction,