Cell growth, cell death and the V-type H[+]-ATPase

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学 位 論 文 要 旨

[Abstract]

Recent studies have shown that bafilomycin A_1-sensitive vacuolar-type H^+-ATPase (V-ATPase) plays important roles in cell growth and tumor progression. V-ATPase is composed of two distinct structures, a hydrophilic catalytic cytosolic sector (V_1) and a hydrophobic transmembrane sector (V_0). The V_1 sector is composed of 5-8 different subunits with the structure $A_3B_3C_1D_1E_1F_1G_1H_1$. The V_0 sector is composed of 5 different subunits with the structure $116_138_119_116_6$. The overexpression of 16-kDa proteolipid subunit of V-ATPase in the perinuclear region of the human adventitial fibroblasts promotes phenotypic modulation that contributes to neointimal formation and medial thickening. A relationship between oncogenicity and the expression of the 16-kDa proteolipid has also been suggested in human pancreatic carcinoma tissue.

We found in this study that the mRNA levels of the 16-kDa proteolipid but not of the 70-kDa subunit of V-ATPase in human myofibroblasts were more abundant in cells cultured in serum-containing medium (MF (+) cells) than in cells cultured in serum-free medium (MF (-) cells). In HeLa cells, the levels of mRNA and protein of the 16-kDa, 21-kDa or 70-kDa were clearly suppressed when the corresponding antisense oligonucleotides were administered to the culture medium. However, the growth rate

and viability (mostly due to necrosis) of HeLa cells were reduced markedly by the 16-kDa and 21-kDa antisense, but little by the 70-kDa antisense, and not at all by any sense oligonucleotides. The localization of 16-kDa/21-kDa proteolipid subunits was different from that of the 70-kDa subunit in HeLa cells.

These results suggest that the 16-kDa and 21-kDa proteolipid subunits of the V_0 sector play crucial roles in the growth and death of cultured human cells. Our results may provide new insights into the mechanism and therapeutic implications for vessel wall hyperplasia and tumorigenesis.

[Introduction]

Eukaryotic cells of animals, plants and fungi are endowed with an intracellular membrane system called the vacuolar system, which consists of lysosomes, endosomes, the Golgi apparatus, and a group of secretory granules including synaptic vesicles, where unique vacuolar type H^+-ATPases (V-ATPases) acidify the intravacuolar space. The proton gradient generated by the H^+-ATPase plays vital roles in maintaining many of the normal functions of endocytic and exocytic pathways. Vacuolar type H^+-ATPases are also bound on the plasma membranes of kidney epithelial cells, osteoclasts and macrophages, where they secrete protons through the plasma membrane, thereby participating in urinary acidification, bone resorption, cytoplasmic pH regulation and K^+ secretion. V-ATPase in the plasma membrane also has critical functions in tumor cells for metastasis and in seminal ducts for spermatogenesis.

V-ATPase is composed of two distinct structures, a hydrophilic catalytic cytosolic sector (V_1) and a hydrophobic transmembrane sector (V_0). The V_1 sector is composed of 5-8 different subunits including a hexamer of three copies each of subunit A (ranging from 65 to 75 kDa) and subunit B (ranging from 55 to 60 kDa) plus accessory subunits C (40-45 kDa), D (32-34 kDa), E (27-31 kDa), F (13-14 kDa), G (12-13 kDa) and H (50-54 kDa), which altogether form an approximately 570 kDa peripheral complex with the structure $A_3B_3C_1D_1E_1F_1G_1H_1$. The membrane integral V_0 sector forms a pathway for

proton conductance and contains subunits of molecular mass 100-116 kDa (subunit a), 38-39 kDa (subunit d), 19-23 kDa (subunit c") and 14-17 kDa (subunit c, c'), and forms an approximately 270 kDa integral complex with the structure $116_1 38_1 19_1 16_6$. Among these subunits, c, c' and c" are highly hydrophobic proteins called proteolipids; all three proteins are homologous to each other and to subunit c of the F_1F_0-ATP synthase (F-ATPase) and contain 4 to 5 putative transmembrane segments. Each of the proteolipid subunits contains an essential carboxyl group buried in membranes that is critical for proton transport. In subunit c of the F-ATPase, this critical carboxyl group is in TM2 whereas in subunits c and c' of the V-ATPase, the critical carboxyl residue is located in TM4. Subunit c" of the V-ATPase contains an essential glutamic acid residue in TM3. Mutation of any of these sites completely abolishes proton transport by the V-ATPase, indicating that each V-ATPase complex must contain at least one copy of each of the proteolipid subunits.

Although the exact function of each subunit is not completely elucidated, the 16-kDa proteolipid subunit is considered to be an essential component of the V_0 membrane channel sector. The 16-kDa proteolipid is a highly conserved family of polypeptides implicated in diverse transport functions in eukaryotic cells.

Recently, interest in the 16-kDa proteolipid of V-ATPase has been growing because similar 16-kDa proteolipids are involved in structures other than H^+-ATPase, such as the gap junctional complexes and mediatophores. It also associates with the E5 oncoprotein and $\beta 1$ integrin. It's overexpression leads to cell transformation through a potential link between receptor signal transduction pathways and membrane pore activity. A positive relationship between oncogenicity and the expression of the 16-kDa proteolipid subunit of V-ATPase has also been suggested in a human pancreatic carcinoma tissue.

Relatively little is known about the 21-kDa proteolipid subunit in contrast to the 16-kDa proteolipid. Furthermore, no evidence for the association of the 21-kDa proteolipid with either the 16-kDa proteolipid or other V-ATPase subunits has been reported in any organism other than yeast or mouse. Anraku's group reported that there are three distinct

proteolipid subunits in yeast V-ATPase: c (Vma3p), c' (Vma11p), and c" (Vma16p). They demonstrated that the three proteolipid subunits exhibit many common properties. The primary structures of these proteins are similar to one another, and all three subunits are essential for activity and assembly of the complex enzyme. Forgac's group reported the sequence of the mouse 21-kDa subunit c" and demonstrated that its expression pattern and intracellular localization are similar to those of the 16-kDa proteolipid subunit, consistent with its function as a subunit of the V_0 domain.

Otani found that the expression of the 16-kDa proteolipid of V-ATPase was stronger in the MF (+) cells (myofibroblasts incubated in serum-containing medium), a model of neointimal hyperplasia after Percutaneous Transluminal Coronary Angioplasty (PTCA), than in the MF (-) cells (myofibroblasts incubated in serum-free medium). That study demonstrated that the 16-kDa subunit of V-ATPase plays a crucial role in vessel wall hyperplasia following growth stimulation.

To investigate the relationship between the subunits of V-ATPase and the growth or death of human cells, we determined the mRNA expression levels of the 16-kDa and 70-kDa subunits of MF (+) cells and of MF (-) cells from human saphenous vein segments. Furthermore, we added the antisense or sense oligodeoxyribonucleotide (oligonucleotide) of the 16-kDa, 21-kDa or 70-kDa subunit of the V-ATPase to the culture medium of HeLa cells and investigated the mRNA and protein levels, the growth and the viability of HeLa cells. We also reported the intracellular localization of 16-kDa/21-kDa proteolipids and 70-kDa subunits.

[Results]

1. Expression of mRNA for the human 16-kDa and 70-kDa subunit of V-ATPase in MF (+) and MF (-) cells: Otani *et al* found that the expression of 16-kDa proteolipid of V-ATPase was stronger in MF(+) cells than in MF(-) cells. We determined the levels of mRNA quantitatively for the human 16-kDa and 70-kDa subunits of V-ATPase in the MF (+) and MF (-) cells using RT-PCR and Southern blotting. The mRNA levels of the

16-kDa subunit were higher in the MF (+) cells than in the MF (-) cells (Fig. 1A), while there was no evident difference in the mRNA expressions of the 70-kDa subunit between these two types of cells (Fig. 1B). β-actin (control) showed no difference in the mRNA expressions between the MF (+) and the MF(-) cells (Fig. 1C). These results suggest that the 16-kDa proteolipid but not the 70-kDa subunit of V-ATPase is overexpressed in the human proliferative saphenous vein myofibroblast cells.

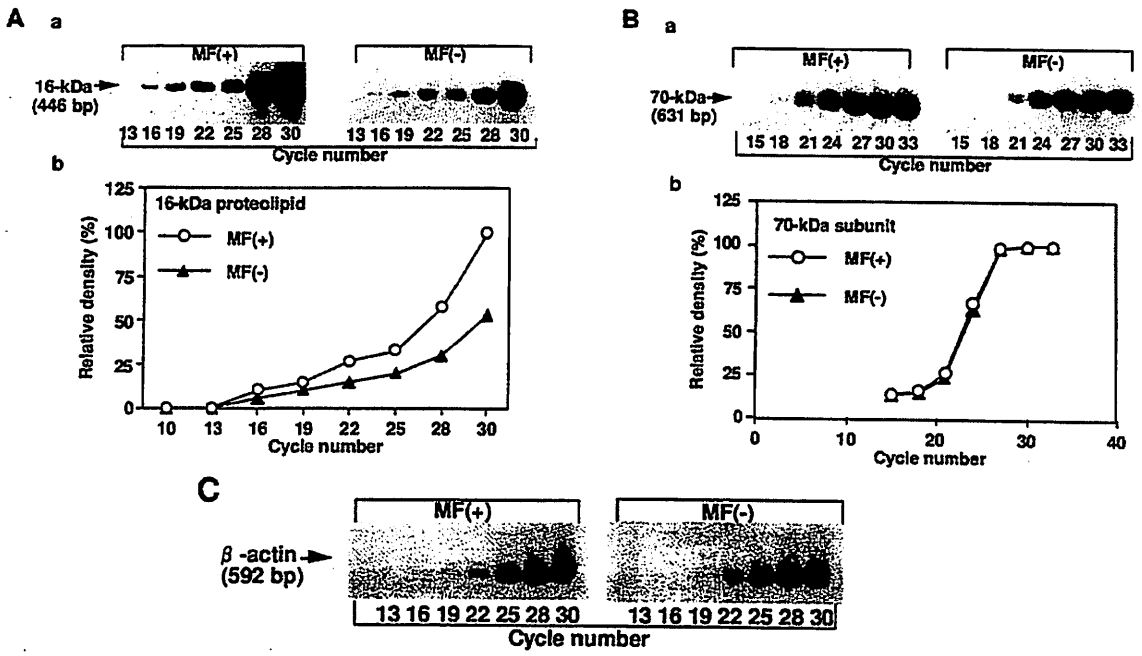
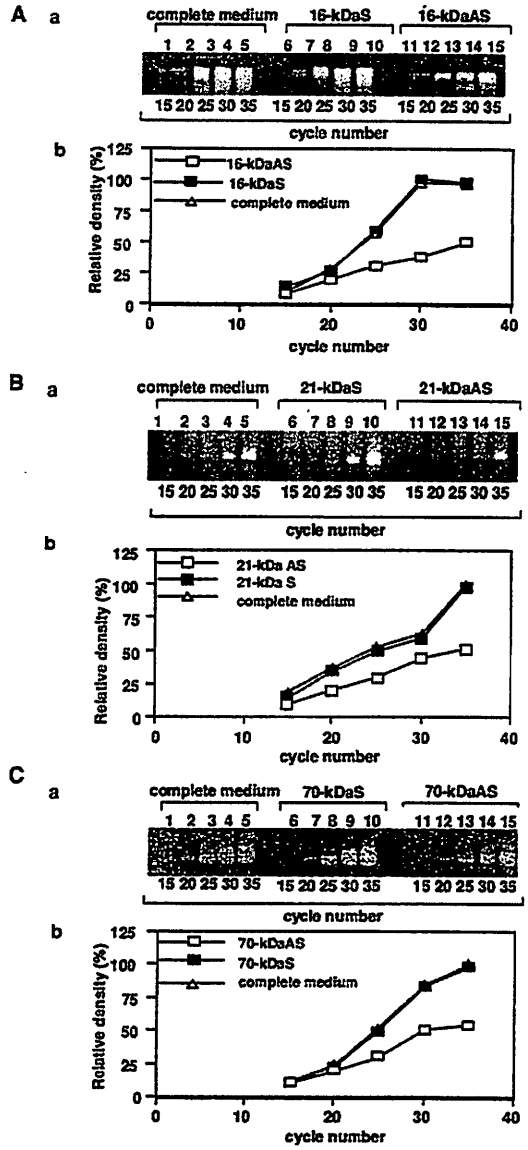


Fig.1 Expression of mRNA for 16-kDa and 70-kDa subunits of V-ATPase in MF(+) and MF(-) cells.

2. Different roles of proteolipids and 70-kDa subunits of V-ATPase in growth and death of cultured human cells

To clarify further the cell growth and death are due to the levels of V-ATPase or just due to the proteolipid subunits of V-ATPase, we determine their functional involvement in HeLa cells using antisense oligonucleotides against 16-kDa, 21-kDa and 70-kDa subunits. We first looked at the effect of blockage of these genes with antisense oligonucleotide on the levels of corresponding mRNA and protein. As shown in furgure, the levels of mRNA (Fig.2) and protein (Fig.3) of the 16-kDa, 21-kDa or 70-kDa were

clearly suppressed when the corresponding antisense oligonucleotides were administered to the culture medium, whereas it was not reduced by sense oligonucleotide. Then, we investigated the growth rate and cell viability of HeLa cells after treatment with antisense



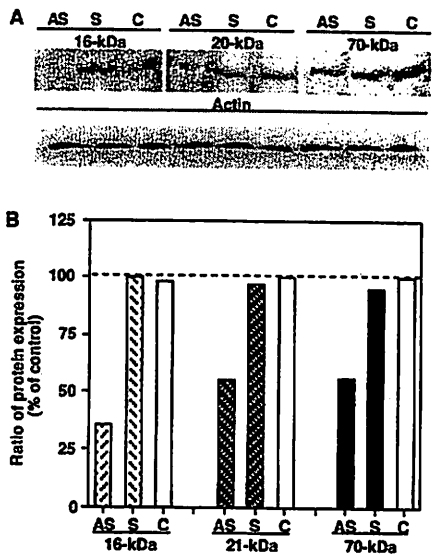
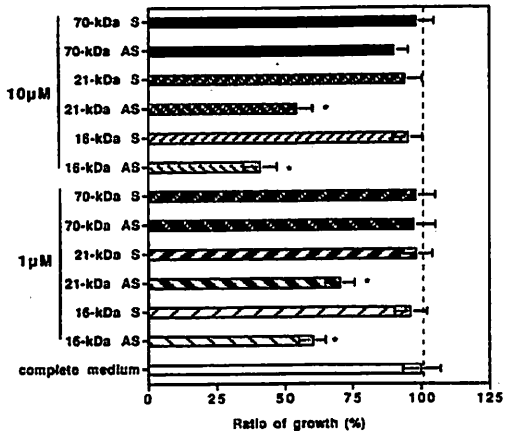


Fig.2 (Left) Effects on mRNA contents of HeLa cells after treatment with antisense or sense oligonucleotide

Fig.3 (Right) Antisense oligonucleotides-induced decrease in target protein

oligonucleotides. We found that the growth rate (Fig.4) and viability (Fig.5) of HeLa cells were reduced markedly by the 16-kDa or 21-kDa antisense oligonucleotide, but little by the 70-kDa antisense or any sense oligonucleotide. Furthermore, we showed that apoptosis was not the leading cause of cell death induced by the 16-kDa or 21-kDa antisense oligonucleotide; although it is one of the pathway (Fig.6).

In order to study the mechanism of the different roles between the 16-kDa/21-kDa proteolipid and 70-kDa subunits, we investigated their intracellular localization. We found



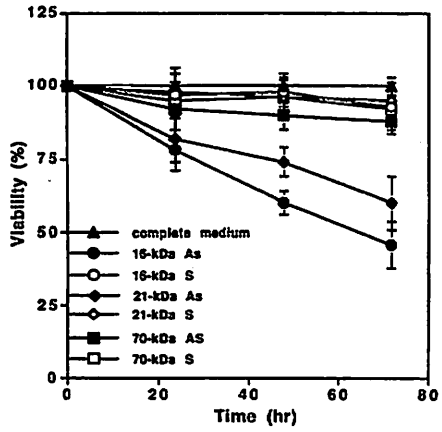
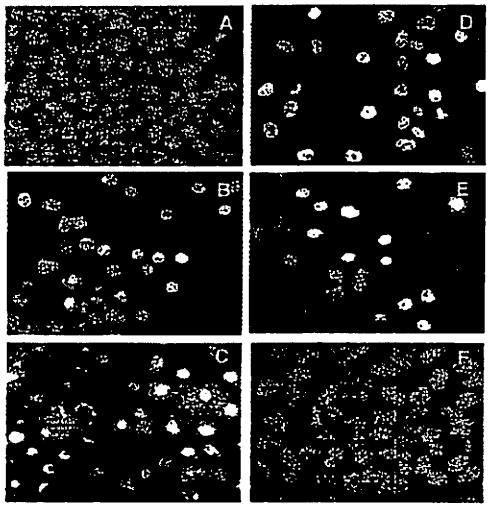


Fig.4 (Left) Effect of 16-kDa, 21-kDa or 70-kDa antisense oligonucleotide on the growth of HeLa cells

Fig.5 (Right) Reduction in the viability of HeLa cells caused by 16-kDa and 21-kDa antisense oligonucleotides



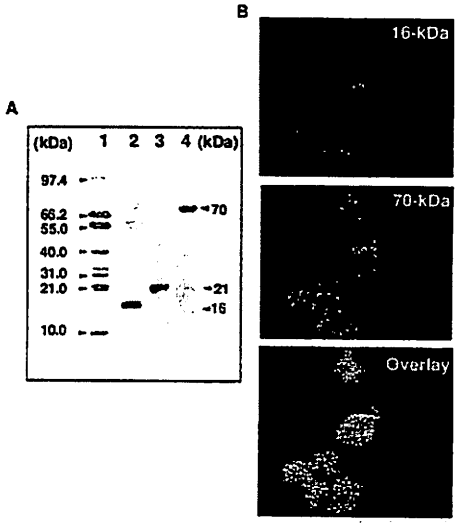


Fig.6 (Left) Morphological features of HeLa cells stained with Hoechest 33258.

(A) complete medium, 72h

(B) 4mM KCN plus 50mM 2-deoxy-D-glucose, 4h

(C) 100mM bnfilomyan A1, 48h (D, E, F)10 μ mol/L 16-kDa, 21-kDa or 70-kDa anti-sence oligo nucleotide, respectively, 72h

Fig.7 (Right) Immunolocalization studies for the 16-kDa proteolipid and 70-kDa subunits.

that the 16-kDa subunit has a similar expression pattern and co-localize with the 21-kDa subunit in HeLa cells, whereas the expressed proteolipid proteins fail to co-localize with the 70-kDa subunit. As shown in Fig.7, staining pattern were perinuclear and punctate in the cytoplasm, with some diffuse and weak staining throughout the remainder of the

HeLa cells, for the 16-kDa proteolipid subunit as compared with 70-kDa subunit.

[Discussion]

The distinct human genes (subunit c: *ATP6C and* subunit c": *ATP6F*) that putatively encode the V-ATPase proteolipid subunits have been identified. The two 70-kDa subunit isoforms of the V_1 sector of V-ATPase (*VA68-type* and *HO68-type*) were cloned and characterized in human osteoclastoma. *VA68* has a ubiquitous expression pattern in many human tissues and cell lines including HeLa cells, whereas *HO68* expression is restricted to human osteoclastoma tissue. In this paper, the primer and antisense oligonucleotide against the 70-kDa subunit were synthesized according to the *VA68* cDNA.

Otani found that the expression of the 16-kDa proteolipid of V-ATPase was stronger in the MF (+) cells than in the MF (-) cells, showing that the 16-kDa subunit V-ATPase plays a crucial role in vessel wall hyperplasia following growth stimulation. In our studies, the mRNA level of the 16-kDa subunit of V-ATPase was evidently higher in the MF (+) than in the MF (-) cells, while no evident difference was found in the 70-kDa mRNA expression. The results indicate that the 16-kDa subunit of V-ATPase was overexpressed in the human proliferative saphenous vein myofibroblast cells.

To clarify further whether the cell growth and the cell death are due to the level of V-ATPase or just the level of proteolipid subunits of V-ATPase, we tested the relationship between the cell growth, the cell death and the proteolipids or 70-kDa subunit of V-ATPase. In the present study, we used the antisense oligonucleotide against 16-kDa, 21-kDa and 70-kDa mRNA to determine functional involvement in HeLa cells. We found

that all of the 16-kDa, 21-kDa and 70-kDa antisense oligonucleotides at 10 μmol/L clearly reduced the levels of target mRNA and protein in HeLa cells over 72 h. The antisense oligonucleotide against the 16-kDa or the 21-kDa subunit also reduced the growth and the viability of HeLa cells, but less so the antisense oligonucleotide against the 70-kDa subunit and not at all any sense oligonucleotides. Our results indicate that the 16-kDa and 21-kDa proteolipids of the V_0 sector were more important than the 70-kDa subunit of the V_1 sector in the growth and the death of HeLa cells. These results are consistent with the higher expression levels of 16-kDa mRNA in the MF (+) cells than in the MF (-) cells. Ohta and Numata also suggested overexpression of the 70-kDa subunit A and 30-kDa subunit E of the V_1 sector in human invasive pancreatic tumor, but less than that of the 16-kDa subunit. Furthermore, we showed that apoptosis was not the only or leading cause of cell death induced by the 16-kDa or 21-kDa antisense oligonucleotide, although it is one of the pathways.

The precise mechanisms of the different roles of proteolipid and 70-kDa subunits of V-ATPase in the growth and death of cultured human cells remain to be clarified. We consider it to be probably related to their expression patterns in tissues and their intracellular localization. We acquired that distinct perinuclear and punctate staining patterns in cytoplasm were observed for the antisera against the 16-kDa proteolipid subunit as compared with the 70-kDa subunit. The co-localization of the 16-kDa and 21-kDa subunits visualized by immunofluorescence is consistent with these proteins being present in the same V_0 complexes and both the 16-kDa and 21-kDa subunits

having similar functions in the growth and survival of HeLa cells. The distinct localization patterns observed in the V_1 and V_0 domains may be the reason for the different functions. Alternatively, the different functions are also probably related to the assembly of V-ATPase. Studies in yeast have indicated that reversible dissociation and reassembly of the V_1 and V_0 domains represents an important mechanism for directly controlling V-ATPase activity. Glucose deprivation has been shown to cause a rapid dissociation of the V-ATPase from approximately 80% to approximately 20%. The free V_1 and V_0 domains have also been identified in the lysosomal and the mammalian cells. The existence of separate pools of V_1 and V_0 domains suggests that this may be a widely employed mechanism. Nelson's group found that of all the V-ATPase subunits only the 16-kDa proteolipid is assembled independently. The 16-kDa proteolipid subunit serves as a template for the assembly of the other subunits. Studies of the yeast V-ATPase indicate that the V_0 domain is able to assemble and target to the central vacuole in the absence of the V_1 subunits, but that in the absence of any of the V_0 subunits both the attachment and the assembly of V_1 is prevented. However, these are just selected possible mechanisms. We can not exclude the possibility of other novel functions of the proteolipid subunits in HeLa cells. Further studies are necessary to clarify the reason for the difference between the 16-kDa/21-kDa proteolipids and 70-kDa subunits.

学位論文審査結果の要旨

V型 H^+ - ATPaseのプロテオリピドは、癌細胞で増加するだけでなく、増殖性大伏在静脈でも増加していることを示唆する結果が出ており、細胞増殖とV - ATPaseとの関係に興味が持たれる。詹（占）紅は、RT - PCR法で、V - ATPaseサブユニットの発現を増殖性大伏在静脈で検討すると共に、V - ATPase遺伝子に対するアンチセンスオリゴヌクレオチドの効果を、HaLa細胞で検討した。

その結果、増殖性大伏在静脈V - ATPaseの、16kDaプロテオリピドmRNAは増加したが、70kDaサブユニットmRNAは殆ど変化しなかった。HeLa細胞V - ATPaseサブユニットの発現は、アンチセンスでいずれも強く押さえられたが、細胞増殖は、16kDaと21kDaプロテオリピドのアンチセンスで抑えられ、細胞死が引き起こされたが、70kDaサブユニットのアンチセンスでは殆ど影響が見られなかった。細胞内局在性は、16kDaと21kDaプロテオリピドで同じであったが、70kDaと21kDaプロテオリピドで同じであったが、70kDaサブユニットでは若干異なった。V - ATPaseサブユニットに対する抗体でも、問題点はあるがアンチセンスと矛盾しないとの予備結果を得た。

詹（占）紅の得たこれらの結果は、細胞増殖・細胞死等に対するV - ATPaseサブユニットの働きに新知見を加えるもので、博士（薬学）に値するものと判定した。