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Identification of Hexapeptides that Render C2 Myoblasts the Resistant to Menadione-induced Cell Death

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Abstract: Menadione induced cell death in cultured C2 myoblasts. By screening synthetic peptide libraries composed of random sequence of hexapeptides, we identified the hexa-peptides pool of (Ala/Ile)-(Ile/Met)-Val-Ile-Asp-(Met/Ser)-NH_2 that protected the myoblasts against menadione-induced cell death. Pre-incubation with the hexapeptide pool reduced the number of cells detached from culture dish substrate and increased the ratio of relative viability against menadione. In addition, the peptides strongly increased the expression of Bcl-2, an anti-apoptotic protein. These results suggest that the hexapeptides might enhance the resistance to cell death against menadione by increasing the expression of Bcl-2.

Key words: C2 myoblasts, menadione, oxidative stress, cell death, hexapeptides, Bcl-2

Skeletal muscle is normally subjected to oxidative stress during exercise because intense contractile activity is associated with increased free radical production (McArdle et al., 2005). Thus, skeletal muscle is highly adapted to reduce redox imbalances. On the other hand, reactive oxygen species (ROS) may contribute to muscle degeneration in many diseases, such as muscular dystrophies and atrophies (Kefaloyianni et al., 2006). Duchenne muscular dystrophy (DMD) is a severe degenerative muscle disease caused by mutations in the gene encoding dystrophin. Because the symptoms of dystrophin deficiency are similar to that of muscle injury due to oxidative stress, it has been postulated that the cell death in muscular dystrophies may be due to free radical-mediated injuries (Rando et al., 1998). Menadione, a quinone-containing compound, is metabolized to semiquinone by flavoprotein reductase, which is then converted back to quinone via oxidation in the presence of molecular oxygen (Monks et al., 1992). Menadione has been shown to induce oxidative stress in skeletal muscle cells (Sun et al., 1997). Menadione-induced cell death occurred in a dose-dependent manner: apoptosis at lower concentrations and necrosis at higher concentrations in C2C12 myoblasts (Chiou et al., 2003).

The amino acid sequence is important for the formation of active site, which plays a key role in the function of a protein. Recently, various methods have been developed to identify sequences of vast mixtures of random peptides (Houghten et al., 1999). One of the most widespread applications was the mixture based positional scanning method (Pinilla et al., 1992). The positional scanning libraries are composed of systematically arranged mixture having defined and mixed positions. Thus, information regarding the activity of each function is obtained for each position of the library (Pinilla et al., 2003). In this study, by screening synthetic peptide libraries composed of random sequence of hexapeptides, we identified hexapeptides pools that enhanced the cells' resistance to cell death and increased the expression of Bcl-2 under menadione-induced oxidative stress in C2 myoblasts.

MATERIALS AND METHODS

Cell culture and viability assay

C2 myoblasts were cultured in Dulbeccos modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator supplied with 5% CO_2. To assay the effect of menadione, myoblasts were preincubated in serum-free DMEM for 1 h, and then exposed to menadione or dimethylsulfoxide (DMSO), the vehicle. The cells were cultured for 5 h after which cell viability was assessed. To determine the effect of peptides on cell viability, cells were pre-incubated for 1 h in the presence of peptide mixtures before menadione was added. Cell viability was determined by using MTT [3-(4,5-

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dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. For the assay, the cells were grown in 96 wellplates and the MTT reagent was added to each well and incubated for 4 h at 37°C. The dark blue formazan crystals were solubilized in DMSO and measured at 570 nm with background subtraction at 655 nm on an ELISA plate reader.

Preparation of positional scanning-synthetic peptide combinatorial libraries (PSSPCLs)

PS-SPCLs were obtained from Peptide Library Support Facility of Pohang University of Science and Technology (South Korea). The 6 sets of PS-SPCLs (total of 114 pools) are represented by the formula: $O_1XXXXX\text{-}NH_2$, $XO_2XXXX\text{-}NH_2$, $XXO_3XXX\text{-}NH_2$, $XXXO_4XX\text{-}NH_2$, $XXXXO_5X\text{-}NH_2$, and $XXXXXO_6\text{-}NH_2$ in which O represents defined positions and X represent the positions with mixed amino acids (Houghten et al., 1991; Park et al., 1997). Each of the 114 peptide mixtures was individually dissolved in distilled water and added to the cells at the final concentration of 1.2 nM. The reiterative hexapeptide mixture, (Ala/Ile)-(Ile/Met)-Val-Ile-Asp-(Met/Ser)-NH2, was also obtained from Peptide Library Support Facility of Pohang University of Science and Technology.

Western blotting

To determine the effect of the hexapeptides pool on Bcl-2 the expression, cells were harvested and disrupted as previously described (Woo and Kim, 2006). The immunoreactive proteins were detected using the enhanced chemiluminescence (ECL) method. The relative band densities were analyzed using Scion-image program.

RESULTS AND DISCUSSION

A novel hexapeptides pool which strengthens resistance against menadioneinduced cell death was identified by screening the PS-SPCLs.

It has previously been reported that menadione induces oxidative stress in skeletal muscle cells (Sun et al.,1997). In the present study, to determine the effective concentration of menadione under our culture condition, growing C2 myoblasts were exposed to menadione at various concentrations and cell viability was measured by MTT assay. As shown in Fig. 1, the cell viability sharply decreased at 10 μM of menadione and most cells were lost at 20 μM. The effective menadione concentration was changed with cell density (data not shown). Repeated experiments showed that the half maximum cell death occurred at around 15 μM menadione. We used this menadione concentration for the subsequent studies.

Peptides that suppress cell death under the menadione-induced oxidative stress were identified by screening of the PS-SPCLs. To determine the most effective amino acid sequence, C2 myoblasts at 70% confluency were pre-incubated for 1 h with each pool of hexapeptides in which a single position was defined to be a specific amino acid (except cysteine). Subsequently the cells were treated with menadione and cultured for 5 h. The results of the initial screening of the peptide library are shown in Fig. 2. Amino acids in each position were selected by their effect on cell viability: Alanine (Ala) or isoleucine (Ile) in the first position, isoleucine (Ile) or methionine (Met) in the second, valine (Val) in the third, isoleucine (Ile) in the fourth, aspartic acid (Asp) in the fifth, and methionine (Met) or serine (Ser) in the sixth positions. Peptides were then synthesized as follows. Positions 3, 4 and 5 consisted of defined amino acids. The remaining three positions 1, 2 and 6 were composed of mixtures of selected amino acids. These generated 8 hexapeptides with mixtures of two amino acids at positions 1, 2 and 6: X1-X2-Val-Ile-Asp-X6-NH2, where X1 was either Ala or Ile, X2 was either Met or Ile and X6 was either Met or Ser. These synthesized peptide pool contained 8 individual hexapeptides; Ala-Ile-Val-Ile-Asp-Met-NH2, Ala-Ile-Val-Ile-Asp-Ser-NH2, Ala-Met-Val-Ile-Asp-Met-NH2, Ala-Met-Val-Ile-Asp-Ser-NH2, Ile-Ile-Val-Ile-Asp-Met-NH2, Ile-Ile-Val-Ile-Asp-Ser-NH2, Ile-Met-Val-Ile-Asp-Met-NH2, and Ile-Met-Val-Ile-Asp-Ser-NH2.

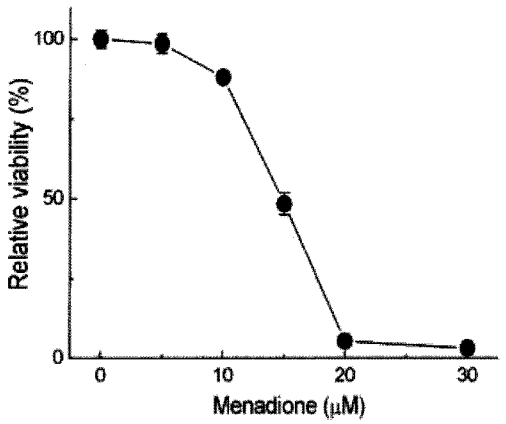

Fig. 1. Cell viability under menadione-induced oxidative stress in C2 myoblasts. Growing C2 myoblasts were pre-incubated in serum-free DMEM for 1 h. The cells were then exposed to DMSO, a vehicle, or menadione at various doses and cultured for 5 h. Cell viability was measured by MTT assay as described in Materials and Methods. Relative viability is expressed as a ratio of each treatment to the DMSO-treated cells.

Novel hexapeptides increased cell survival and Bcl-2 expression under menadione-induced oxidative stress.

To assess the effects of hexapeptides, we first observed cell morphology under the microscope. It was previously reported that menadione induced both apoptosis and necrosis in cultured C2C12 muscle cells depending on its concentration

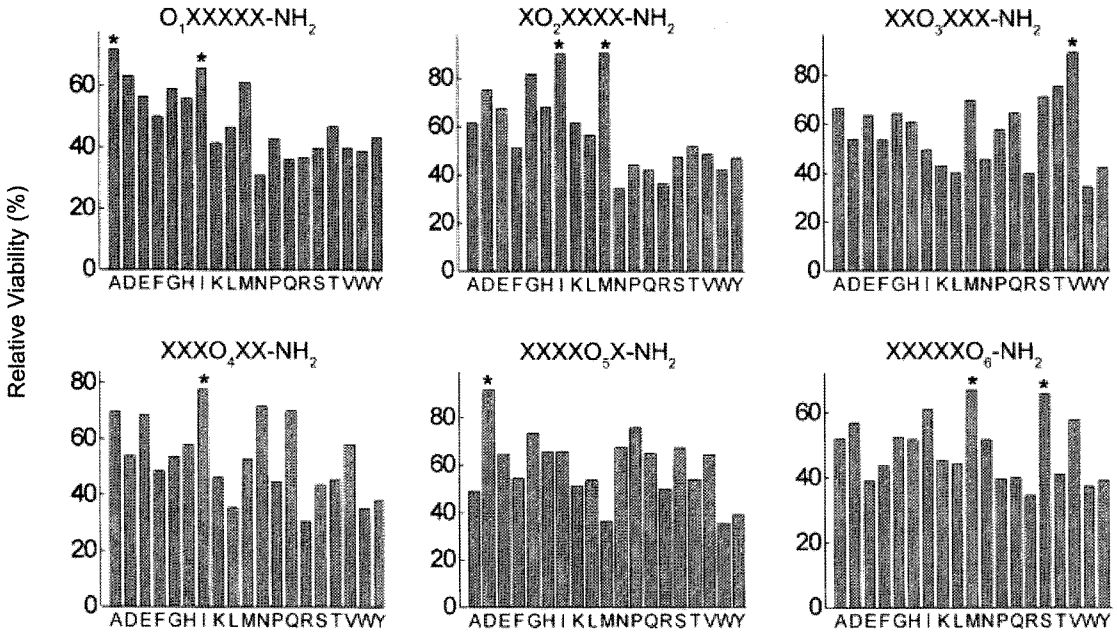

Fig. 2. Screening of PS-SPCLs for peptides that render cells resistant to menadione-induced oxidative stress. C2 myoblasts were pre-incubated in serum-free DMEM containing each of 114 hexapeptides that have defined amino acid at each position (cysteine was excluded) for 1 h. The cells were then exposed to 15 μM menadione for 5 h. Cell viability was measured by MTT assay. The defined amino acids are represented on the X-axis by their single letter codes. The most effective amino acids were marked by asterisks (*).

(Chiou et al., 2003). During apoptosis, cells undergo shrinkage, and become rounded and detached from the substrate of culture dish (Rudolf and Cervinka, 2006). As shown in Fig. 3A, incubation with 15 μM menadione increased the number of rounded cells and vacant areas were formed in the culture dish. However, pre-incubation with 100 μM hexapeptides mixture of (Ala/Ile)-(Ile/Met)-Val-Ile-Asp-(Met/Ser)-NH2 prevented their phenomenon. To insure the effect of the hexapeptides on cell survival, myoblasts were exposed to menadione with various concentrations of the hexapeptides. About 60% in the menadione-treated cells were viable compared to vehicle-treated cells. However, when cells were pre-incubated with the hexapeptides mixture, the percentage of viable cells slightly increased compared to vehicle-treated control (Fig. 3B).

Bcl-2 is a well-known anti-apoptotic protein (Haddad, 2004). It is Known to be down-regulated during apoptosis (Burlacu, 2003; Dominov et al., 2005), and its enhanced expression suppressed the apoptosis by oxidative stress (Metrailler-Ruchonnet et al., 2007). We examined whether the hexapeptide mixture might affect the expression of Bcl-2. As shown in Fig. 3C, the hexapeptides significantly increased the expression of Bcl-2 in a dose-dependent manner. These results indicated that the hexapeptides might block the menadione-induced cell death in part, through induction of Bcl-2 expression. Apoptosis significantly contributes to pathogenesis of muscular dystrophies. It has been reported that over-expression of Bcl-2 could inhibit the apoptosis in muscular dystrophies including DMD (Basset et al., 2006; Girgenrath et al., 2004). Because the hexapeptide mixture strongly increased the expression of Bcl-2 in myoblasts under oxidative stress, it might be applied as a therapeutic tool to protect cells against oxidative stress and to inhibit the progression of apoptosis in muscular dystrophies. Further study of each synthetic peptide would be helpful in identification of drug targets in oxidative stress-induced muscle damage of muscular dystrophy.

Because synthetic peptides can not efficiently traverse the plasma membrane except for some peptides such as cell penetrating peptides (CPP) (Wagstaff and Jans, 2006), we cannot expect that the hexapeptides mixture directly enter the cells. Instead, the hexapeptides may activate cell survival signaling pathway(s) via receptors on the plasma membrane. The pathways which are activated under oxidative stress and/or are upstream of Bcl-2 expression include phosphoinositide 3-kinase (PI3-kinase), nuclear factor B (NF-κB), p38 mitogen-activated protein kinase (MAPK), and Jun N-terminal kinase (JNK) (Chen et al., 1998; Niwa et al., 2003; Shimada et al., 2003). To clarify the action mechanism of the hexapeptides, the relationship with those signaling pathways will be further studied.

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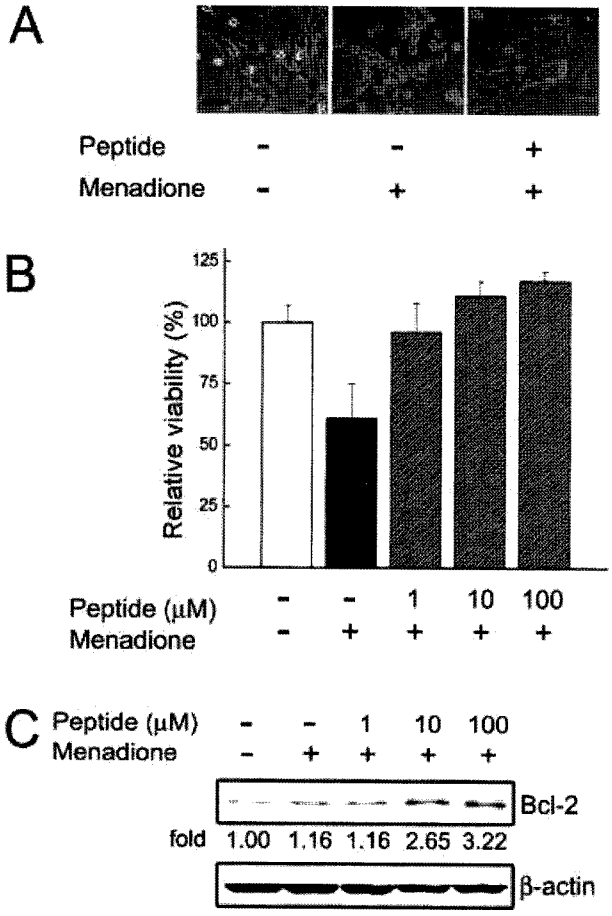

Fig. 3. Effect of hexapeptide mixture of (Ala/Ile)-(Ile/Met)-Val-Ile-Asp-(Met/Ser)-NH_2 on cell survival against menadione. C2 myoblasts were pre-incubated in serum-free DMEM containing the hexapeptide mixture for 1 h and then with 15 μM menadione for 5 h. (A) The cells cultured with 100 μM of the hexapeptide mixture were observed under a phase-contrast microscope. Bar indicates 100 μm. (B) Cell viability was measured by MTT assay. The relative viability is expressed as a ratio of each treatment to the DMSO-treated cells. (C) The expression of Bcl-2 was determined by western blotting. β-Actin was used as a loading control.

REFERENCES

Basset O, Boittin FX, Cognard C, Constantin B, and Ruegg UT (2006) Bcl-2 overexpression prevents calcium overload and subsequent apoptosis in dystrophic myotubes. *Biochem J* 395:267-276.

Burlacu A (2003) Regulation of apoptosis by Bcl-2 family proteins. *J Cell Mol Med* 7:249-257.

Chen YR, Wang W, Kong AN, and Tan TH (1998) Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates. *J Biol Chem* 273:1769-1775.

Chiou TJ, Chu ST, and Tzeng WF (2003) Protection of cells from menadioneinduced apoptosis by inhibition of lipid peroxidation. *Toxicology* 191:77-88.

Dominov JA, Kravetz AJ, Ardelt M, Kostek CA, Beermann ML, and Miller JB (2005) Muscle-specific BCL2 expression ameliorates muscle disease in laminin {alpha}2-deficient, but not in dystrophin-deficient, mice. *Hum Mol Genet* 14:1029-1040.

Girgenrath M, Dominov JA, Kostek CA, and Miller JB (2004) Inhibition of apoptosis improves outcome in a model of congenital muscular dystrophy. *J Clin Invest* 114:1635-1639.

Haddad JJ (2004) On the antioxidant mechanisms of Bcl-2: a retrospective of NF-kappaB signaling and oxidative stress. *Biochem Biophys Res Commun* 322:355-363.

Houghten RA, Pinilla C, Appel JR, Blondelle SE, Dooley CT, Eichler J, Nefzi A, and Ostresh JM (1999) Mixture-based synthetic combinatorial libraries. *J Med Chem* 42:3743-3778.

Houghten RA, Pinilla C, Blondelle SE, Appel JR, Dooley CT, and Cuervo JH (1991) Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature* 354:84-86.

Kefaloyianni E, Gaitanaki C, and Beis I (2006) ERK1/2 and p38-MAPK signalling pathways, through MSK1, are involved in NF-kappaB transactivation during oxidative stress in skeletal myoblasts. *Cell Signal* 18:2238-2251.

McArdle F, Pattwell DM, Vasilaki A, McArdle A, and Jackson MJ (2005) Intracellular generation of reactive oxygen species by contracting skeletal muscle cells. *Free Radic Biol Med* 39:651-657.

Metrailler-Ruchonnet I, Pagano A, Carnesecchi S, Ody C, Donati Y, Barazzone and Argiroffo C (2007) Bcl-2 protects against hyperoxia-induced apoptosis through inhibition of the mitochondria-dependent pathway. *Free Radic Biol Med* 42:1062-1074.

Monks TJ, Hanzlik RP, Cohen GM, Ross D, and Graham DG (1992) Quinone chemistry and toxicity. *Toxicol Appl Pharmacol* 112:2-16.

Niwa K, Inanami O, Yamamori T, Ohta T, Hamasu T, and Kuwabara M (2003) Redox regulation of PI3K/Akt and p53 in bovine aortic endothelial cells exposed to hydrogen peroxide. *Antioxid & Redox Signaling* 5:713-722.

Park JY, Kim IJ, Lee MH, Seo JK, Suh PG, Cho BY, Ryu SH, and Chae CB (1997) Identification of the peptides that inhibit the stimulation of thyrotropin receptor by Graves' immunoglobulin G from peptide libraries. *Endocrinology* 138:617-626.

Pinilla C, Appel JR, Blanc P, and Houghten RA (1992) Rapid identification of high affinity peptide ligands using positional scanning synthetic peptide combinatorial libraries. *Biotechniques* 13:901-905.

Pinilla C, Appel JR, Borras E, and Houghten RA (2003) Advances in the use of synthetic combinatorial chemistry: mixture-based libraries. *Nat Med* 9:118-122.

Rando TA, Disatnik MH, Yu Y, and Franco A (1998) Muscle cells from mdx mice have an increased susceptibility to oxidative stress. *Neuromuscul Disord* 8:14-21.

Rudolf E, and Cervinka M (2006) Cytoskeletal changes in non-apoptotic cell death. *Acta Medica (Hradec Kralove)* 49:123-128.

Shimada K, Nakamura M, Ishida E, Kishi M, and Konishi N (2003) Roles of p38- and c-jun NH2-terminal kinase-mediated pathways in 2-methoxyestradiol-induced p53 induction and apoptosis. *Carcinogenesis* 24:1067-1075.

Sun JS, Tsuang YH, Huang WC, Chen LT, Hang YS, and Lu FJ (1997) Menadione-induced cytotoxicity to rat osteoblasts. *Cell Mol Life Sci* 53:967-976.

Wagstaff KM, and Jans DA (2006) Protein transduction: cell penetrating peptides and their therapeutic applications. *Curr Med Chem* 13:1371-1387.

Woo JH, and Kim HS (2006) Phosphorylation of eukaryotic elongation factor 2 can be regulated by phosphoinositide 3-kinase in the early stages of myoblast differentiation. *Mol Cells* 21:294-301.

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