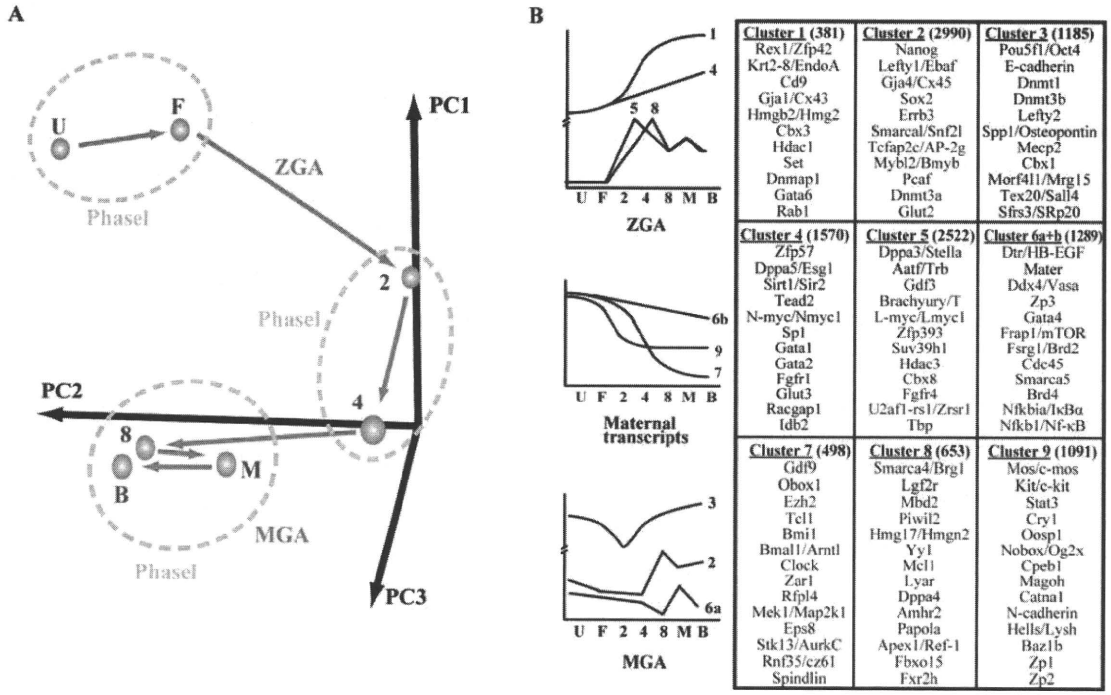

Cluster 1 (381)	Cluster 2 (2990)	Cluster 3 (1185)
Rex1/Zfp42	Nanog	Pou5f1/Oct4
Krt2-8/EndoA	Lefty1/Ebaf	E-cadherin
Cd9	Gja4/Cx45	Dnmt1
Gja1/Cx43	Sox2	Dnmt3b
Hmgb2/Hmg2	Errb3	Lefty2
Cbx3	Smarcal/Snf2l	Spp1/Osteopontin
Hdac1	Tcfap2c/AP-2g	Mecp2
Set	Mybl2/Bmyb	Cbx1
Dnmap1	Pcaf	Morf4l1/Mrg15
Gata6	Dnmt3a	Tex20/Sall4
Rab1	Glut2	Sfrs3/SRp20
Cluster 4 (1570)	**Cluster 5 (2522)**	**Cluster 6a+b (1289)**
Zfp57	Dppa3/Stella	Dtr/HB-EGF
Dppa5/Esg1	Aatf/Trb	Mater
Sirt1/Sir2	Gdf3	Ddx4/Vasa
Tead2	Brachyury/T	Zp3
N-myc/Nmyc1	L-myc/Lmyc1	Gata4
Sp1	Zfp393	Frap1/mTOR
Gata1	Suv39h1	Fsrg1/Brd2
Gata2	Hdac3	Cdc45
Fgfr1	Cbx8	Smarca5
Glut3	Fgfr4	Brd4
Racgap1	U2af1-rs1/Zrsr1	Nfkbia/IκBα
Idb2	Tbp	Nfkb1/Nf-κB
Cluster 7 (498)	**Cluster 8 (653)**	**Cluster 9 (1091)**
Gdf9	Smarca4/Brg1	Mos/c-mos
Obox1	Lgf2r	Kit/c-kit
Ezh2	Mbd2	Stat3
Tcl1	Piwil2	Cry1
Bmi1	Hmg17/Hmgn2	Oosp1
Bmal1/Arntl	Yy1	Nobox/Og2x
Clock	Mcl1	Cpeb1
Zar1	Lyar	Magoh
Rfpl4	Dppa4	Catna1
Mek1/Map2k1	Amhr2	N-cadherin
Eps8	Papola	Hells/Lysh
Stk13/AurkC	Apex1/Ref-1	Baz1b
Rnf35/cz61	Fbxo15	Zp1
Spindlin	Fxr2h	Zp2

Fig. (1). Expression Profiling of Preimplantation Embryos.

A. Principal component analysis based on the expression data by gene chip array.

A matrix of scatter plots. U, F, 2, 4, 8, M, and B denote unfertilized egg, fertilized egg, 2-cell embryo, 4-cell embryo, 8-cell embryo, morula, and blastocyst, respectively. Each scatter plot shows the comparison of gene expression between embryo stages.

B. Expression changes of individual genes analyzed by the k-means non-hierarchical clustering method. Gene expression patterns can be assigned to three main groups. Group 1 (Cluster 1, 4, 5 and 8) appears to represent ZGA genes that are first activated from the zygotic genome. Group 2 (Cluster 7 and 9) represents maternal transcripts with distinctive patterns of degradation during preimplantation development. Group 3 (Cluster 2 and 3) appears to represent genes that follow a combination of these two patterns.

(Modified from Hamatani *et al.* Dev Cell, 2004, 6, 117 [18]).

embryos. Recent progress in RNA amplification methods using *in vitro* transcription and microarray platforms, including genes unique to preimplantation embryos, allows us to apply global gene expression profiling to the study of preimplantation embryos [17]. Hamatani *et al.* reported, for the first time, the global gene expression profiles of preimplantation embryos at all stages [18]. More than half of 21,939 gene features show statistically significant changes during preimplantation development. Pair-wise comparison, hierarchical clustering analysis, and principal component analysis (PCA) reveal two major transient waves of de novo transcription as follows: the first wave corresponds to zygotic genome activation (ZGA); the second wave, mid-preimplantation gene activation (MGA), contributes dramatic morphological changes during late preimplantation development (Fig. (**1A**)). Unsupervised methods such as principal component analysis (PCA) can transform the original features into new features (principal components (PC)), each PC representing a linear combination of the original features. PCA reduces input dimensionality by providing a subset of components that captures most of the information in the original data. For example, those genes that are highly correlated with the most informative PCs could be selected as classifier inputs, rather than a large dimension of original variables containing redundant features. To trace the expression changes of individual genes, statistically significant genes are analyzed by the k-means non-hierarchical clustering method. Gene expression patterns of these clusters can be assigned to three main groups (Fig. (**1B**)). The first group appears to represent ZGA genes that are first activated from the zygotic genome (Fig. (**1B**) Clusters 1, 4, 5, and 8). According to Gene Ontology (GO) terms [19] by MAPPFinder [20, 21], ZGA is suggested not to be promiscuous and to contribute mainly to the preparation of basic cellular machinery during the 2-cell and the 4-cell stages. The second group represents maternal transcripts with distinctive patterns of degradation during preimplantation development (Fig. (**1B**) Clusters 7 and 9). The third group appears to represent genes that follow a combination of these two patterns (Fig. (**1B**) Clusters 2 and 3). Genes whose expression first significantly increases from the 4-cell to 8-cell stage are identified as MGA genes, of which there are 4,216. The functional assignment of these genes by MAPPFinder characterizes the function of the MGA genes by the following three representative GO terms: "endopeptidase inhibitor," "intercellular junction," and "DNA (cytosine-5-)-methyltransferase." The implication of these GO terms and the timing of MGA seems consistent with the proposed role of MGA in compaction, cavitation, and the first differentiation of ICM and TE. Expression profiling of embryos treated

with inhibitors of transcription and translation reveals that the translation of maternal RNAs is required for the initiation of ZGA, suggesting a cascade of gene activation from maternal RNA/protein sets to ZGA gene sets and thence to MGA gene sets.

Decreasing oocyte competence with maternal aging is a major concern in human infertility because the rate of late childbearing is increasing even though reproductive capacity in women declines dramatically with advancing age. Studies of molecular mechanisms involved in the decline of oocyte quality with maternal age could have important implications for the efficacy and safety of clinical ooplasmic donation to rejuvenate aging oocytes. Hamatani *et al.* and Pan *et al.* also reported age-associated alteration of gene expression patterns in mouse oocytes, which has implications for aging research [22, 23]. Genes related to oxidative stress (e.g., Sod1, Apacd and Txn1), mitochondrial function (e.g., Sdha, Pdhb and Cyb5), chromatin structure (e.g., Hdac2, Hmgb3 and Bmi1), DNA methylation (e.g., Dnmt1, Dnmt3b, and Dnmt3L), and genome stability (e.g., Tert, Exo1, and Msh3) are altered with aging. Furthermore, kinetochore components of the spindle assembly checkpoint (e.g., Bub1, BubR1, Aurora kinase) and Cdc20, a critical activator of the Anaphase Promoting Complex, may contribute to aneuploidy in aged oocytes [23].

These comprehensive expression profiles of the majority of genes should give a baseline for analysis of the complex gene regulatory networks in normal mouse preimplantation and for comparative analysis for other mammalian species, including humans.

WHAT'S GOING ON IN AN EPITHELIAL-MESENCHYMAL TRANSITION (EMT)?

The conversion of an epithelial cell to a mesenchymal cell is critical to vertebrate embryogenesis and a defining structural feature of organ development, such as forming fibroblasts in injured tissues [24, 25], or in initiating metastases in epithelial cancer [26-29]. From a general perspective, EMT is about disaggregating epithelial units and reshaping epithelia for movement. Epithelia in transition lose polarity, tight junctions, adherens junctions, desmosomes and cytokeratin intermediate filaments in order to rearrange their F-actin stress fibers and express lamellopodia.and filopodia. This phenotypic conversion requires the molecular reprogramming of epithelia with new biochemical instructions. It is known that commonly used molecular markers for EMT include increased expression of N-cadherin and vimentin, nuclear localization of beta-catenin, and increased production of the transcription factors such as Snail, Twist, and SIP1/ZEB2. Much of this conversion, however, has been studied during experiments that expose new transduction and signaling pathways in epithelia, and more recently in fibrogenic tissues. It is not yet clear whether the fibroblast transition of EMT is an expected middle phase of transdifferentiating epithelia, or whether EMT producing fibroblasts is an arrested form of transdifferentiation.

EMT is easily engaged by a combination of cytokines associated with proteolytic digestion of basement membranes upon which epithelia reside. We analyzed PCA and hierarchical clustering method of the gene expression pattern of the renal tubular cells and mammary gland cells. If PC1 were used to identify genes that are differentially expressed between phenotypes, then genes that are strongly associated with PC1 would be selected. If both PC axes are used, then genes strongly associated with two groups would be selected. We then identified the genes which discriminate between the renal tubular and the mammary gland epithelial cells (PC1), or EMT-induced and non-induced cells (PC3) (Fig. (**2**)). Undergoing EMT identifies the genes that discriminate between the renal tubular and the mammary gland epithelial cells (PC1), or EMT-induced and non-induced cells (PC3) (Table **1**).

The advanced study of the genes identified by PCA would yield new insight regarding EMT, and achieve a breakthrough in understanding the molecular mechanisms of

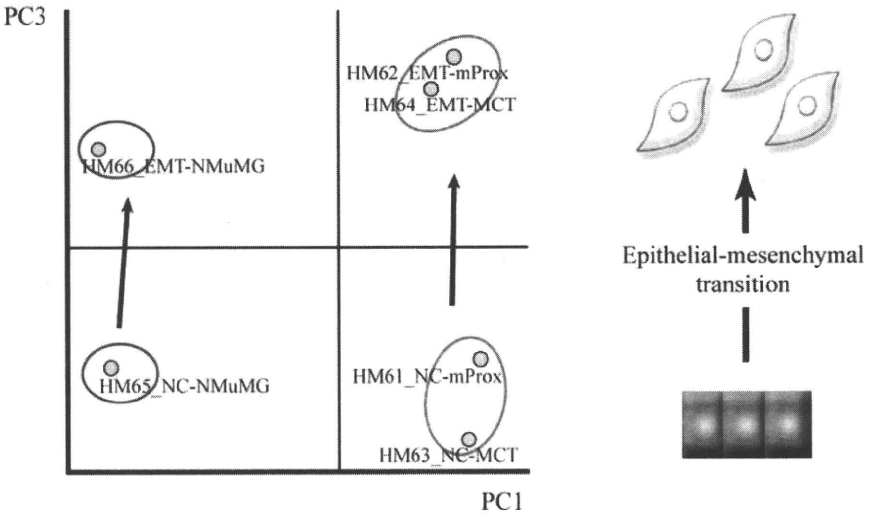

Fig. (2). EMT related genes by gene chip analysis.

Principal component analysis based on the gene expression pattern of the renal tubular cells and mammary gland cells.

Table 1. List of Genes that Had Up-Regulated (Positive) and Down-Regulated (Negative) Expression Related to EMT (PC3 Axis)

Positive			Negative		
Gene Symbol	**Gene Title**	**Representative Public ID**	**Gene Symbol**	**Gene Title**	**Representative Public ID**
Ccng2	cyclin G2	U95826	Bach1	BTB and CNC homology 1	NM_007520
Ccni	cyclin I	NM_017367	Cdc42bpa	Cdc42 binding protein kinase alpha	BM117074
Ctgf	connective tissue growth factor	NM_010217	Dnm1	dynamin 1	L31397
Dock7	Dedicator of cytokinesis 7	BB463580	Foxb1	forkhead box B1	U90538
Dok1	docking protein 1	BC006868	Gprc5c	G protein-coupled receptor, family C, group 5, member C	BC008228
Fgfr1	Fibroblast growth factor receptor 1	M33760	Il13ra1	interleukin 13 receptor, alpha 1	S80963
Gja1	gap junction membrane channel protein alpha 1	BB039269	Kcnk5	potassium channel, subfamily K, member 5	AF319542
Gtpbp4	GTP binding protein 4	AI987834	Kif13a	kinesin family member 13A	AB037923
Hgfac	hepatocyte growth factor activator	NM_019447	Kif17	kinesin family member 17	AW492270
Hoxa3	homeo box A3	BB496114	Mark2	MAP/microtubule affinity-regulating kinase 2	BI686265
Hoxb8 /b7	homeo box B8 / homeo box B7	X13721	Mef2d	myocyte enhancer factor 2D	NM_133665
Il15ra	interleukin 15 receptor, alpha chain	NM_008358	Mrpl51	mitochondrial ribosomal protein L51	AI594880
Irx2	Iroquois related homeobox 2	AF295369	Mxd4	Max dimerization protein 4	BE291523
Itga5	integrin alpha 5	BB493533	Neu1	neuraminidase 1	AI649303
Itgb1	integrin beta 1	BM120341	Rel	reticuloendotheliosis oncogene	NM_009044
Mapkbp 1	Mitogen activated protein kinase binding protein 1	BQ174980	Rgnef	Rho-guanine nucleotide exchange factor	BG069493
Mdm2	transformed mouse 3T3 cell double minute 2	X58876	Rps6kb2	ribosomal protein S6 kinase, polypeptide 2	NM_021485
Ncam1	neural cell adhesion molecule 1	NM_010875	Slc24a1	solute carrier family 24, member 1	BC016094
Pdgfa	platelet derived growth factor, alpha	BB371842	Slc25a19	solute carrier family 25, member 19	AV338420
Prkcc	protein kinase C, gamma	NM_011102	Slc25a22	solute carrier family 25 , member 22	AK018760
Rab23	RAB23, member RAS oncogene family	NM_008999	Slc40a1	solute carrier family 40 , member 1	AF226613
Rasa3	RAS p21 protein activator 3	NM_009025	Stat1	signal transducer and activator of transcription 1	AW214029
Rb1	retinoblastoma 1	NM_009029	Tgfa	transforming growth factor alpha	M92420
Sbno1	Sno, strawberry notch homolog 1	BC023136	Ubp1	upstream binding protein 1	NM_013699
Slc1a4	solute carrier family 1, member 4	BB277461	Usp12	ubiquitin specific protease 12	AF441835
Slc34a1	solute carrier family 34 , member 1	AI788646			
Slc4a7	Solute carrier family 4, member 7	AW555750			
Slc7a2	solute carrier family 7, member 2	M62838			
Ube1y1	ubiquitin-activating enzyme E1, Chr Y 1	X62581			
Vegfa	vascular endothelial growth factor A	NM_009505			
Wnt6	wingless-related MMTV integration site 6	NM_009526			
Wnt7b	wingless-related MMTV integration site 7B	W29605			

drug delivery for specific anti-cancer drugs, especially those affecting metastasis. Progress in understanding EMT has been an exercise in coming to appreciate the level of complexity required for changing cellular identity. The mecha-

nism of EMT highlights an integration of nuclear regulation and network signaling with alterations in the microenvironment to create a moving cell; in this sense, basic concepts based on EMT mechanisms would thus hold great promise for regenerative medicine.

GET OP9 CELLS DEAD TO RIGHTS

The concept of regenerative medicine refers to the cell-mediated restoration of damaged or diseased tissue. Candidate cell sources for tissue regeneration include ES cells, fetal cells, and adult cells such as marrow stromal cells, each of which has both advantages and drawbacks. Clinical trials with marrow stromal cells have been performed in patients with osteogenesis imperfecta and osteoporosis, and marrow stromal cells are expected to be a good source of cell therapy.

Bone marrow-derived stem cells can be transdifferentiated into multilineage cells, such as muscle [30] from mesoderm, lung [31] and liver [31, 32] from endoderm, and brain [33-36] and skin [31] from ectoderm. Somatic stem cells are more desirable than ES cells for cell therapeutics because of ethical considerations and the possible immunologic rejection of ES cells. Mesenchymal stem cells have become the most popular somatic stem cells in medicine and biology, not least because of their high reproductive capability *in vitro*.

Chondrocytes differentiate from mesenchymal cells during embryonic development [37], and the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan [38-40]. The phenotype of differentiated chondrocytes is rapidly lost since it is unstable in culture [41-44]. This process is referred to as 'dedifferentiation' and is a major impediment to the use of mass cell populations for therapy or tissue engineering of damaged cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocytes morphologically transform into flattened fibroblast-like cells, with profound changes in biochemical and genetic characteristics, including reduced synthesis of type II collagen and cartilage proteins [45].

We established several stromal cells from murine bone marrow cultures [46]. One of them, KUSA-A1 cells, displays osteogenetic characteristics *in vitro* and *in vivo*. In order to clarify the specific gene expression profile of KUSA-A1, other established stromal cells, KUM5, 9-15c, KUSA-O, H-1/A [47], and mouse embryonic fibroblasts, we compared the expression levels of approximately 23,000 genes by using the Affymetrix gene chip oligonucleotide arrays. Of the 23,000 genes represented on the gene chip, chondrocyte-specific or -associated genes such as type II collagen α1, type XI collagen α1, Sox9, proline arginine-rich end leucine-rich repeat, and cartilage oligomeric matrix protein are more strongly expressed in KUM5 cells than in other marrow-derived mesenchymal cells. Does a gene expression pattern reflect the character of the cells *in vitro* and/or *in vivo*? – The answer is yes: KUM5 cells generate hyaline cartilage and exhibit endochondral ossification *in vitro* (Fig. (**3B**)) and *in vivo* [48].

Surprisingly, OP9 cells [49] also express these chondrocyte-specific or -associated genes at higher levels: the type II collagen α1, and type XI collagen α1 genes are expressed in OP9 cells at more than 10-fold higher levels than in 9-15c, KUSA-O, H-1/A, primary embryonic fibroblasts, or even KUM5 chondroblasts. In addition, expression of 'structural proteins' on Gene Ontology, including the extracellular matrix, is much higher by OP9 and KUM5 cells than by non-chondrogenic cells such as KUSA-A1, H-1/A, and 9-15c, implying that the OP9 and KUM5 cells are mainly engaged in synthesizing extracellular matrix. We also performed hierarchical clustering and PCA, based on the microarray data (Fig. (**3A**)). KUM5 and OP9 cells are grouped into the same subcategory and can clearly be separated from other stromal cells based on the expression data of cell surface markers and cell-type-specific genes, implying that KUM5 and OP9 cells have chondrogenic potential.

Are OP9 cells chondroblasts *in vitro* and/or *in vivo*? - the answer, again, is yes; OP9 cells are induced into the chondrogenic lineage by the pellet culture method (Fig. (**3C**)), and the OP9 pellets (micromasses) implanted in mice form the type II collagen-positive hyaline cartilage [48]. OP9 cells are derived from macrophage colony-stimulating factor-deficient osteopetrotic mice, and have also been used as feeder cells for embryonic stem cells [50-52]. The cells identified as a key participant in regulating the number of adult stem cells or hematopoietic stem cells are now considered to be of an osteoblastic lineage [53, 54]. OP9 cells have been recognized as a niche-constituting preadipocyte; however their true face is a chondroblast. We have two different types of cells, osteoblasts (KUSA-A1) and chondroblasts (OP9 and KUM5), showing distinctive *in vivo* characteristics. The unique characteristics of these cells provide an opportunity to analyze the process of membranous ossification and endochondral ossification. These cells are useful candidate cell sources, in addition to dedifferentiated chondrocytes obtained from cartilage for transplantation in osteoarthritis and rheumatoid arthritis.

GENE EXPRESSION PROFILING AND MEDICAL SCIENCE

Recently, gene expression profiling has been successfully used to predict outcomes in some types of malignant diseases [55-61] and, additionally, to assess drug discovery screening [62]. In reproductive and regenerative medicine, it is important to identify biomarkers that will establish the isolation, selection and expansion of stem cells *in vitro* to allow their use for cell therapy. On the road map for translational medicine-- often referred to as bench to bedside research--, stem cell therapy is a prime destination. Stem cells have not taken on the identity of any specific cell type and are not yet committed to any dedicated function; they can divide extensively or indefinitely, and may be induced to give rise to one or more specialized cell types. Stem cells derived from bone marrow can replace heart muscle lost as a result of a heart attack, and can improve cardiac function. Injecting bone-marrow stem cells into an injured heart potentially represented new therapy, triggering the launch of numerous clinical studies to investigate the effect of directly injecting these cells into the damaged heart muscle of patients following a

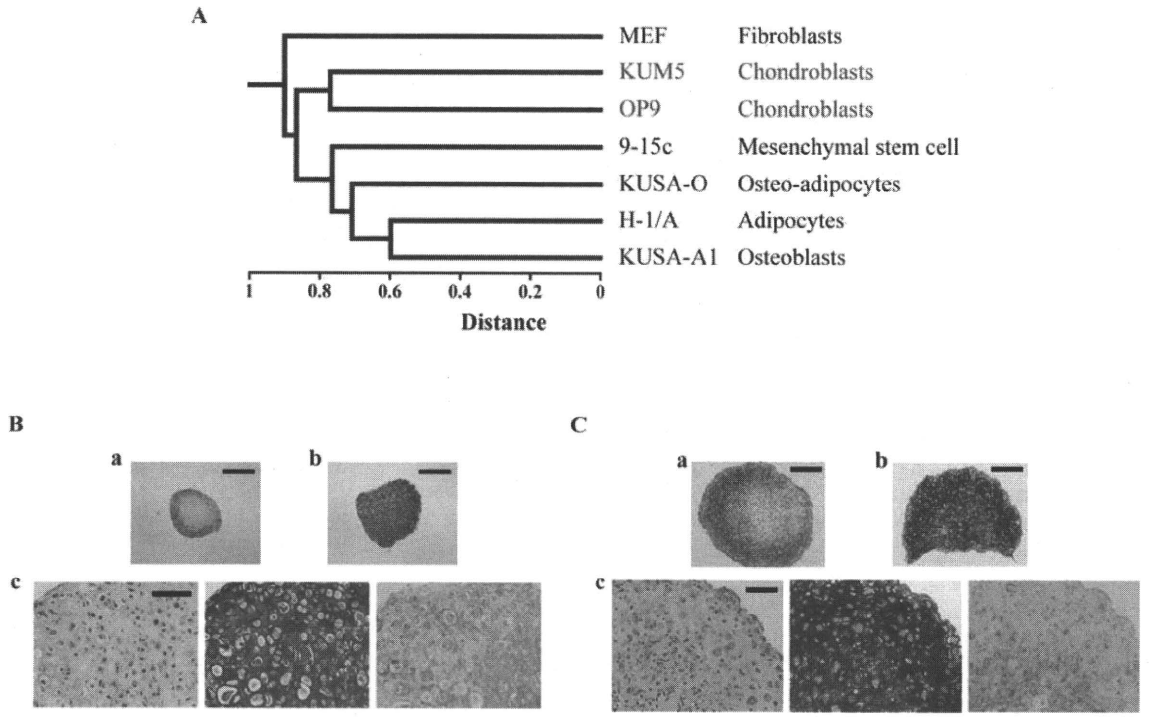

Fig. (3). Expression profiling and *in vitro* chondrogenesis of KUM5 and OP9 cells.

A. Dendrogram revealing clustering profile of six marrow stromal cells and mouse embryonic fibroblast (MEF) using 244 surface marker genes.

B. a, b: Toluidine blue stained section of KUM5 chondrogenic nodules in the pellet culture exposed TGF-β3 and BMP-2 for 1 (a) or 3 (b) weeks. Scale bars: 500 μm. c: Higher magnification of KUM5 chondrogenic pellet exposed to TGF-β3 and BMP-2 for 3 weeks. Left panel: hematoxylin and eosin stain; center panel: toluidine blue stain; right panel: alcian blue stain. Scale bars: 100 μm.

C. a, b: Toluidine blue stained section of OP9 chondrogenic nodules in the pellet culture exposed TGF-β3 and BMP-2 for 1 (a) or 3 (b) weeks. Scale bars: 500 μm. c: Higher magnification of OP9 chondrogenic pellet exposed to TGF-β3 and BMP-2 for 3 weeks. Left panel: hematoxylin and eosin stain; center panel: toluidine blue stain; right panel: alcian blue stain. Scale bars: 100 μm.

(Modified from Sugiki *et al.* J Cell Biochem, 2007, 100, 1240 [48]).

heart attack [63]. The scientific underpinnings of the ongoing human studies have been established. Now is the time to search for the presence of naturally occurring, authentic pluripotent cells and to identify and dissect the signals that guide their migration, self-renewal and differentiation. Furthermore, we need to commit the necessary time and resources to identify the best stem cells for cell therapy to translate.

CONCLUSION

Here, the expression pattern has been correlated with molecular structure descriptor; this consistency indicates that the expression profiling is valid. Consequently, understanding the global gene network that governs the pluripotency and self-renewal of stem cells is an important first step towards the experimental manipulation of cellular developmental potency. The cell potency is a fundamental concept in developmental biology and stem cell biology, providing a conceptual framework of sequential transition from totipotent fertilized eggs to pluripotent embryonic stem cells and stem cells to terminal differentiated cells. The global expression profiling can help to delineate the global architecture and dynamics of a gene regulatory network such as Oct4-regulated gene networks in mouse ES cells [64].

ACKNOWLEDGEMENTS

The authors wish to thank Yoriko Takahashi for helpful discussions, and Kayoko Saito for secretarial assistance.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan; the Ministry of Health, Labour and Welfare Sciences Research Grants; by a Research Grant on Health Science focusing on Drug Innovation from the Japan Health Science Foundation; by the program for promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency; by a Research Grant for Cardiovascular Disease from the ministry of Health, Labour and Welfare; and by a Grant for Child Health and Development from the Ministry of Health, Labour and Welfare.

FOOTNOTE

Fig. (**1**) is prepared from ref. [18] with permission from Elsevier.

Fig. (**3**) is prepared from ref. [48] with permission from Wiley-Liss, Inc.

REFERENCES

[1] Bryder, D.; Rossi, D.J.; Weissman, I.L. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am. J. Pathol.,* **2006**, *169*, 338-346.

[2] Alison, M.; Sarraf, C. Hepatic stem cells. *J. Hepatol.,* **1998**, *29*, 676-682.

[3] Watt, F.M. Epidermal stem cells: markers, patterning and the control of stem cell fate. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* **1998**, *353*, 831-837.

[4] Potten, C.S. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* **1998**, *353*, 821-830.

[5] Gage, F.H. Mammalian neural stem cells. *Science,* **2000**, *287*, 1433-1438.

[6] Jiang, Y.; Vaessen, B.; Lenvik, T.; Blackstad, M.; Reyes, M.; Verfaillie, C.M. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp. Hematol.,* **2002**, *30*, 896-904.

[7] Jiang, Y.; Jahagirdar, B.N.; Reinhardt, R.L.; Schwartz, R.E.; Keene, C.D.; Ortiz-Gonzalez, X.R.; Reyes, M.; Lenvik, T.; Lund, T.; Blackstad, M.; Du, J.; Aldrich, S.; Lisberg, A.; Low, W.C.; Largaespada, D.A.; Verfaillie, C.M. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature,* **2002**, *418*, 41-49.

[8] Fridenshtein, A. [Stromal bone marrow cells and the hematopoietic microenvironment]. *Arkh. Patol.,* **1982**, *44*, 3-11.

[9] Ko, M.S. Embryogenomics: developmental biology meets genomics. *Trends Biotechnol.,* **2001**, *19*, 511-518.

[10] Ivanova, N.B.; Dimos, J.T.; Schaniel, C.; Hackney, J.A.; Moore, K.A.; Lemischka, I.R. A stem cell molecular signature. *Science,* **2002**, *298*, 601-604.

[11] Ramalho-Santos, M.; Yoon, S.; Matsuzaki, Y.; Mulligan, R.C.; Melton, D.A. "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science,* **2002**, *298*, 597-600.

[12] Schena, M.; Shalon, D.; Davis, R.W.; Brown, P.O. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science,* **1995**, *270*, 467-470.

[13] Tanaka, T.S.; Kunath, T.; Kimber, W.L.; Jaradat, S.A.; Stagg, C.A.; Usuda, M.; Yokota, T.; Niwa, H.; Rossant, J.; Ko, M.S. Gene expression profiling of embryo-derived stem cells reveals candidate genes associated with pluripotency and lineage specificity. *Genome Res.,* **2002**, *12*, 1921-1928.

[14] Brown, P.O.; Botstein, D. Exploring the new world of the genome with DNA microarrays. *Nat. Genet.,* **1999**, *21*, 33-37.

[15] DeRisi, J.L.; Iyer, V.R.; Brown, P.O. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science,* **1997**, *278*, 680-686.

[16] Iyer, V.R.; Eisen, M.B.; Ross, D.T.; Schuler, G.; Moore, T.; Lee, J.C.; Trent, J.M.; Staudt, L.M.; Hudson, J., Jr.; Boguski, M.S.; Lashkari, D.; Shalon, D.; Botstein, D.; Brown, P.O. The transcriptional program in the response of human fibroblasts to serum. *Science,* **1999**, *283*, 83-87.

[17] Carter, M.G.; Hamatani, T.; Sharov, A.A.; Carmack, C.E.; Qian, Y.; Aiba, K.; Ko, N.T.; Dudekula, D.B.; Brzoska, P.M.; Hwang, S.S.; Ko, M.S. In situ-synthesized novel microarray optimized for mouse stem cell and early developmental expression profiling. *Genome Res.,* **2003**, *13*, 1011-1021.

[18] Hamatani, T.; Carter, M.G.; Sharov, A.A.; Ko, M.S. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell,* **2004**, *6*, 117-131.

[19] Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T.; Harris, M.A.; Hill, D.P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J.C.; Richardson, J.E.; Ringwald, M.; Rubin, G.M.; Sherlock, G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.,* **2000**, *25*, 25-29.

[20] Dahlquist, K.D.; Salomonis, N.; Vranizan, K.; Lawlor, S.C.; Conklin, B.R. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat. Genet.,* **2002**, *31*, 19-20.

[21] Doniger, S.W.; Salomonis, N.; Dahlquist, K.D.; Vranizan, K.; Lawlor, S.C.; Conklin, B.R. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol,* **2003**, *4*, R7.

[22] Hamatani, T.; Falco, G.; Carter, M.G.; Akutsu, H.; Stagg, C.A.; Sharov, A.A.; Dudekula, D.B.; VanBuren, V.; Ko, M.S. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum. Mol. Genet.,* **2004**, *13*, 2263-2278.

[23] Pan, H.; Ma, P.; Zhu, W.; Schultz, R.M. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev. Biol.,* **2008**, *316*, 397-407.

[24] Strutz, F.; Okada, H.; Lo, C.W.; Danoff, T.; Carone, R.L.; Tomaszewski, J.E.; Neilson, E.G. Identification and characterization of a fibroblast marker: FSP1. *J. Cell Biol.,* **1995**, *130*, 393-405.

[25] Iwano, M.; Plieth, D.; Danoff, T.M.; Xue, C.; Okada, H.; Neilson, E.G. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.,* **2002**, *110*, 341-350.

[26] Janda, E.; Lehmann, K.; Killisch, I.; Jechlinger, M.; Herzig, M.; Downward, J.; Beug, H.; Grunert, S. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J. Cell Biol.,* **2002**, *156*, 299-313.

[27] Kiemer, A.K.; Takeuchi, K.; Quinlan, M.P. Identification of genes involved in epithelial-mesenchymal transition and tumor progression. *Oncogene,* **2001**, *20*, 6679-6688.

[28] Vincent-Salomon, A.; Thiery, J.P. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. *Breast Cancer Res,* **2003**, *5*, 101-106.

[29] Xue, C.; Plieth, D.; Venkov, C.; Xu, C.; Neilson, E.G. The gatekeeper effect of epithelial-mesenchymal transition regulates the frequency of breast cancer metastasis. *Cancer Res.,* **2003**, *63*, 3386-3394.

[30] Ferrari, G.; Cusella-De Angelis, G.; Coletta, M.; Paolucci, E.; Stornaiuolo, A.; Cossu, G.; Mavilio, F. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science,* **1998**, *279*, 1528-1530.

[31] Krause, D.S.; Theise, N.D.; Collector, M.I.; Henegariu, O.; Hwang, S.; Gardner, R.; Neutzel, S.; Sharkis, S.J. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell,* **2001**, *105*, 369-377.

[32] Petersen, B.E.; Bowen, W.C.; Patrene, K.D.; Mars, W.M.; Sullivan, A.K.; Murase, N.; Boggs, S.S.; Greenberger, J.S.; Goff, J.P. Bone marrow as a potential source of hepatic oval cells. *Science,* **1999**, *284*, 1168-1170.

[33] Sanchez-Ramos, J.; Song, S.; Cardozo-Pelaez, F.; Hazzi, C.; Stedeford, T.; Willing, A.; Freeman, T.B.; Saporta, S.; Janssen, W.; Patel, N.; Cooper, D.R.; Sanberg, P.R. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp. Neurol.,* **2000**, *164*, 247-256.

[34] Woodbury, D.; Schwarz, E.J.; Prockop, D.J.; Black, I.B. Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.,* **2000**, *61*, 364-370.

[35] Kohyama, J.; Abe, H.; Shimazaki, T.; Koizumi, A.; Nakashima, K.; Gojo, S.; Taga, T.; Okano, H.; Hata, J.; Umezawa, A. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation,* **2001**, *68*, 235-244.

[36] Kim, B.J.; Seo, J.H.; Bubien, J.K.; Oh, Y.S. Differentiation of adult bone marrow stem cells into neuroprogenitor cells *in vitro*. *Neuroreport,* **2002**, *13*, 1185-1188.

[37] Solursh, M. Differentiation of cartilage and bone. *Curr. Opin. Cell Biol.,* **1989**, *1*, 989-994.

[38] Hauselmann, H.J.; Fernandes, R.J.; Mok, S.S.; Schmid, T.M.; Block, J.A.; Aydelotte, M.B.; Kuettner, K.E.; Thonar, E.J. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J. Cell Sci.,* **1994**, *107 (Pt 1)*, 17-27.

[39] Reginato, A.M.; Iozzo, R.V.; Jimenez, S.A. Formation of nodular structures resembling mature articular cartilage in long-term primary cultures of human fetal epiphyseal chondrocytes on a hydrogel substrate. *Arthritis Rheum.,* **1994**, *37*, 1338-1349.

[40] Archer, C.W.; McDowell, J.; Bayliss, M.T.; Stephens, M.D.; Bentley, G. Phenotypic modulation in sub-populations of human articular chondrocytes *in vitro*. *J. Cell Sci.,* **1990**, *97 (Pt 2)*, 361-

371.

[41] Benya, P.D.; Padilla, S.R.; Nimni, M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell,* **1978**, *15*, 1313-1321.

[42] Benya, P.D.; Shaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell,* **1982**, *30*, 215-224.

[43] Lefebvre, V.; Peeters-Joris, C.; Vaes, G. Production of collagens, collagenase and collagenase inhibitor during the dedifferentiation of articular chondrocytes by serial subcultures. *Biochim. Biophys. Acta,* **1990**, *1051*, 266-275.

[44] Bonaventure, J.; Kadhom, N.; Cohen-Solal, L.; Ng, K.H.; Bourguignon, J.; Lasselin, C.; Freisinger, P. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. *Exp. Cell Res.,* **1994**, *212*, 97-104.

[45] Yoon, Y.M.; Kim, S.J.; Oh, C.D.; Ju, J.W.; Song, W.K.; Yoo, Y.J.; Huh, T.L.; Chun, J.S. Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J. Biol. Chem.,* **2002**, *277*, 8412-8420.

[46] Matsumoto, S.; Shibuya, I.; Kusakari, S.; Segawa, K.; Uyama, T.; Shimada, A.; Umezawa, A. Membranous osteogenesis system modeled with KUSA-A1 mature osteoblasts. *Biochim. Biophys. Acta,* **2005**, *1725*, 57-63.

[47] Umezawa, A.; Maruyama, T.; Segawa, K.; Shadduck, R.K.; Waheed, A.; Hata, J. Multipotent marrow stromal cell line is able to induce hematopoiesis *in vivo*. *J. Cell. Physiol.,* **1992**, *151*, 197-205.

[48] Sugiki, T.; Uyama, T.; Toyoda, M.; Morioka, H.; Kume, S.; Miyado, K.; Matsumoto, K.; Saito, H.; Tsumaki, N.; Takahashi, Y.; Toyama, Y.; Umezawa, A. Hyaline cartilage formation and enchondral ossification modeled with KUM5 and OP9 chondroblasts. *J. Cell. Biochem.,* **2007**, *100*, 1240-1254.

[49] Nakano, T.; Kodama, H.; Honjo, T. *In vitro* development of primitive and definitive erythrocytes from different precursors. *Science,* **1996**, *272*, 722-724.

[50] Kodama, H.; Nose, M.; Niida, S.; Nishikawa, S. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp. Hematol.,* **1994**, *22*, 979-984.

[51] Nakano, T.; Kodama, H.; Honjo, T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science,* **1994**, *265*, 1098-1101.

[52] Nakano, T. Lymphohematopoietic development from embryonic stem cells *in vitro*. *Semin. Immunol.,* **1995**, *7*, 197-203.

[53] Calvi, L.M.; Adams, G.B.; Weibrecht, K.W.; Weber, J.M.; Olson, D.P.; Knight, M.C.; Martin, R.P.; Schipani, E.; Divieti, P.; Bringhurst, F.R.; Milner, L.A.; Kronenberg, H.M.; Scadden, D.T. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature,* **2003**, *425*, 841-846.

[54] Zhang, J.; Niu, C.; Ye, L.; Huang, H.; He, X.; Tong, W.G.; Ross, J.; Haug, J.; Johnson, T.; Feng, J.Q.; Harris, S.; Wiedemann, L.M.; Mishina, Y.; Li, L. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature,* **2003**, *425*, 836-841.

[55] Alizadeh, A.A.; Eisen, M.B.; Davis, R.E.; Ma, C.; Lossos, I.S.; Rosenwald, A.; Boldrick, J.C.; Sabet, H.; Tran, T.; Yu, X.; Powell, J.I.; Yang, L.; Marti, G.E.; Moore, T.; Hudson, J., Jr.; Lu, L.; Lewis, D.B.; Tibshirani, R.; Sherlock, G.; Chan, W.C.; Greiner, T.C.; Weisenburger, D.D.; Armitage, J.O.; Warnke, R.; Levy, R.; Wilson, W.; Grever, M.R.; Byrd, J.C.; Botstein, D.; Brown, P.O.; Staudt, L.M. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature,* **2000**, *403*, 503-511.

[56] Beer, D.G.; Kardia, S.L.; Huang, C.C.; Giordano, T.J.; Levin, A.M.; Misek, D.E.; Lin, L.; Chen, G.; Gharib, T.G.; Thomas, D.G.; Lizyness, M.L.; Kuick, R.; Hayasaka, S.; Taylor, J.M.; Iannettoni, M.D.; Orringer, M.B.; Hanash, S. Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat. Med.,* **2002**, *8*, 816-824.

[57] Pomeroy, S.L.; Tamayo, P.; Gaasenbeek, M.; Sturla, L.M.; Angelo, M.; McLaughlin, M.E.; Kim, J.Y.; Goumnerova, L.C.; Black, P.M.; Lau, C.; Allen, J.C.; Zagzag, D.; Olson, J.M.; Curran, T.; Wetmore, C.; Biegel, J.A.; Poggio, T.; Mukherjee, S.; Rifkin, R.; Califano, A.; Stolovitzky, G.; Louis, D.N.; Mesirov, J.P.; Lander, E.S.; Golub, T.R. Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature,* **2002**, *415*, 436-442.

[58] Rosenwald, A.; Wright, G.; Chan, W.C.; Connors, J.M.; Campo, E.; Fisher, R.I.; Gascoyne, R.D.; Muller-Hermelink, H.K.; Smeland, E.B.; Giltnane, J.M.; Hurt, E.M.; Zhao, H.; Averett, L.; Yang, L.; Wilson, W.H.; Jaffe, E.S.; Simon, R.; Klausner, R.D.; Powell, J.; Duffey, P.L.; Longo, D.L.; Greiner, T.C.; Weisenburger, D.D.; Sanger, W.G.; Dave, B.J.; Lynch, J.C.; Vose, J.; Armitage, J.O.; Montserrat, E.; Lopez-Guillermo, A.; Grogan, T.M.; Miller, T.P.; LeBlanc, M.; Ott, G.; Kvaloy, S.; Delabie, J.; Holte, H.; Krajci, P.; Stokke, T.; Staudt, L.M. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N. Engl. J. Med.,* **2002**, *346*, 1937-1947.

[59] Shipp, M.A.; Ross, K.N.; Tamayo, P.; Weng, A.P.; Kutok, J.L.; Aguiar, R.C.; Gaasenbeek, M.; Angelo, M.; Reich, M.; Pinkus, G.S.; Ray, T.S.; Koval, M.A.; Last, K.W.; Norton, A.; Lister, T.A.; Mesirov, J.; Neuberg, D.S.; Lander, E.S.; Aster, J.C.; Golub, T.R. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. *Nat. Med.,* **2002**, *8*, 68-74.

[60] van 't Veer, L.J.; Dai, H.; van de Vijver, M.J.; He, Y.D.; Hart, A.A.; Mao, M.; Peterse, H.L.; van der Kooy, K.; Marton, M.J.; Witteveen, A.T.; Schreiber, G.J.; Kerkhoven, R.M.; Roberts, C.; Linsley, P.S.; Bernards, R.; Friend, S.H. Gene expression profiling predicts clinical outcome of breast cancer. *Nature,* **2002**, *415*, 530-536.

[61] Yeoh, E.J.; Ross, M.E.; Shurtleff, S.A.; Williams, W.K.; Patel, D.; Mahfouz, R.; Behm, F.G.; Raimondi, S.C.; Relling, M.V.; Patel, A.; Cheng, C.; Campana, D.; Wilkins, D.; Zhou, X.; Li, J.; Liu, H.; Pui, C.H.; Evans, W.E.; Naeve, C.; Wong, L.; Downing, J.R. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell,* **2002**, *1*, 133-143.

[62] Scherf, U.; Ross, D.T.; Waltham, M.; Smith, L.H.; Lee, J.K.; Tanabe, L.; Kohn, K.W.; Reinhold, W.C.; Myers, T.G.; Andrews, D.T.; Scudiero, D.A.; Eisen, M.B.; Sausville, E.A.; Pommier, Y.; Botstein, D.; Brown, P.O.; Weinstein, J.N. A gene expression database for the molecular pharmacology of cancer. *Nat. Genet.,* **2000**, *24*, 236-244.

[63] Chien, K.R. Stem cells: lost in translation. *Nature,* **2004**, *428*, 607-608.

[64] Matoba, R.; Niwa, H.; Masui, S.; Ohtsuka, S.; Carter, M.G.; Sharov, A.A.; Ko, M.S. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. *PLoS One,* **2006**, *1*, e26.

FORUM

Trend Analysis of Research on Informed Consent in Clinical Trials : Comprehensive Retrieval via Electronic Databases

Taichi HATTA[*1], Toshinori MURAYAMA[*1,*2], Keiichi NARITA[*2], Eriko SUMI[*1,*2] and Masayuki YOKODE[*1,*2]

[*1] Department of Clinical Innovative Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan
[*2] Translational Research Center, Kyoto University Hospital, Kyoto, Japan

Informed consent (IC) is an essential ethical requirement for conducting clinical trials. This study aimed to investigate changes in research on IC for clinical trials and compare the trend in Japan with that overseas.

In February 2010, the electronic databases, PubMed and Japana Centra Revuo Medicina were searched for studies on IC for clinical trials both in and outside Japan.

The literature search identified 89 Japanese studies and 401 overseas studies concerning IC for clinical trials. In Japan, these articles were first published in 1993, and the number increased remarkably from 2002. Many of these articles discussed the contribution of a clinical research coordinator or the understanding of the information by trial subjects. The overseas studies were first published in the early 1980s, and the number increased remarkably in the 1990s.

Since, the enforcement of the new Good Clinical Practice in 1998, observational studies on IC for clinical trials have been conducted in Japan. However, most of these studies applied a quantitative approach, and qualitative analysis is limited. In Japan, IC is not focused exclusively on the protection of trial subjects, but also serves as the basis for an important encounter to build the physician-patient relationship. Therefore, investigations of the IC process by qualitative and quantitative approaches are necessary.

Key words : informed consent, clinical trial, research ethics

1. Introduction

Informed consent (IC) is an essential ethical requirement for conducting clinical trials. Originating in the US, the concept of IC was introduced in Japan in the 1980s[1]. Initially, this concept failed to take full root because of its novelty, given the different medical and cultural backgrounds. In the 1990s, research suggested that the physician-patient relationship should be discussed in order to study the nature of the IC process in Japan[2]. However, before the social/cultural environment was fully matured, IC was legally required for the conduct of clinical trials according to the adoption of ICH-GCP in 1998[3]. In the modern era of increasing globalization, the historical and cultural backgrounds have to be reviewed so as to understand the problems surrounding IC in Japan. This will also inevitably lead to discussions about the methodology of observational study for IC.

The objectives of this study were to investigate the trends in investigations on IC for clinical trials, to review the studies on IC for clinical trials in Japan, and to compare the IC studies in Japanese clinical trials with those overseas.

2. Methods

1) Search of literature : Comprehensive retrieval via electronic databases

To examine the number of published papers on IC for clinical trials, we searched PubMed and Japana Centra Revuo Medicina (http://login.jamas.or.jp/enter.html), the latter being an electronic search engine for medical journals written in Japanese. Searches were originally conducted in December 2007, and repeated in February 2010.

To search for Japanese papers on IC for clinical trials, Japana Centra Revuo Medicina was searched using the retrieval terms "informed consent", "clinical trials" and "data collection", and PubMed was searched using "informed consent", "clinical trials", "data collection" and "Japan". Meanwhile, to search for overseas publications, PubMed was searched using the retrieval terms "in-

Address for correspondence : Murayama T. Translational Research Center, Kyoto University Hospital, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

(Manuscript received March 31, 2010 ; revised July 16, 2010 and October 4, 2010 ; accepted October 24, 2010)

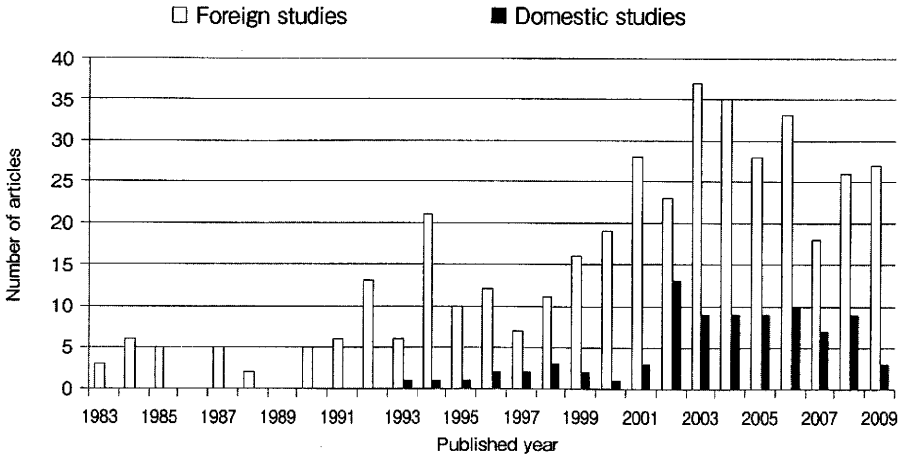

Fig. The number of IC studies for clinical trials

formed consent", "clinical trials" and "data collection", and those identified in the search of Japanese papers were excluded.

2) Extraction and investigation of observational studies on IC in Japan

Japanese studies with abstracts were checked to see whether they were observational studies on IC for participation in clinical trials, which used a questionnaire or an interview with patients, doctors or co-medicals. IC studies without abstracts were excluded because the contents could not be screened, and also most of them were conference proceedings or reviews. Moreover, studies related to decision making in general medicine or studies containing only discussion on the significance of IC for clinical research were also excluded. Then, the selected papers were classified according to the theme of the article and the method used in the study.

3. Results

1) Number of articles identified under each of the search conditions by year

Comprehensive retrieval via electronic databases identified 89 Japanese studies. There was no duplication of data between Japana Centra Revuo Medicina and PubMed. Four hundred and one overseas studies were identified. Figure shows the studies categorized by year of publication.

Japanese research was first reported in the 1990s, and the number increased greatly from 2002. On the other hand, overseas studies were first published in the early 1980s, and the number increased remarkably in the 1990s (Fig.).

2) Extraction and investigation of observational studies on IC in Japan

Thirty-two of 89 Japanese studies had abstracts. Among the 32 articles, 17 that reported observational studies on IC for participation in clinical trials using a questionnaire or an interview of patients, doctors or co-medicals were extracted[4-20]. Most of them dealt with IC for trials for new drug application (NDA).

Six of the 17 articles reported on 'the contribution of a clinical trials coordinator (CRC) in clinical trials'[4,6,11-13,16], 6 reported on IC assessment, such as 'the level of understanding of clinical trials'[7,9,10,14,15,20], and the others studied aspects of IC for clinical trials, such as preference of physicians to recruit participants[5,17], perception of pediatric nurses with regard to competency[8], use of videotape instruction for clinical trials[18] and communication training for CRC[19].

Articles on 'the contribution of a CRC in clinical trials' revealed the factors that participants and physicians recognized as merits in the clinical trials and their impressions of a CRC[4,6,11-13,16]. These articles reported that a CRC contributed mainly to communication with participants, such as drug administration guidance[4], care for participants[4,11] and help in decision-making processes[12,13]. Other articles described the role of a CRC or the expectations of physicians on a CRC[6,16].

Articles on 'the level of understanding of clinical trials' reported how well trial participants understood the clinical trials after receiving an explanation[7,9,10,14,15,20]. The responses suggested that most of the participants could understand the trials if they were given a full explanation. The aspects cited by the participants as difficult to

understand were 'the significance of a placebo' [10], 'double-blinded study' [10,14], 'randomized controlled study' [14] and 'main effect and side effects' [7,9,10,14,15,20].

When the 17 articles were categorized according to study method, the vast majority adopted a quantitative approach, while only one employed a qualitative approach[12]. Nakamura et al. took the verbatim record of IC communication between patients and CRCs, and conducted content analysis, which is a standard methodology in social sciences for studying the content of communication[12].

4. Discussion

1) Relation between domestic IC articles and Japanese social background

We included both articles and conference proceedings (with abstracts) on IC in the present analysis. Although the significance of each type of reference remains to be discussed, we considered them to be equal in this trend analysis. The rationale is that our purpose was to investigate the trends of research on IC for clinical trials, and if conference proceedings were excluded, the retrieved data might be too small to be analyzed properly and the trend might be biased.

In this study, a search of literature using electronic databases found that research on IC for clinical trials had been published in Japan since 1993, and the number increased remarkably from 2002. Many articles concerning IC for clinical trials discussed the contribution of a CRC or the understanding of information provided to trial subjects. These results might be related to the Japanese social background factors such as regulation and infrastructure regarding clinical trials. Specifically, it was a major task for institutions to train CRCs initially, and CRC developed rapidly as a new occupation to support clinical trials including IC.

Although IC started to be practiced substantially in Japan in the early 1980s[1], this study indicates that the focus on IC for clinical trials started later. One of the possible reasons for this delay is that the procedure of IC for general medical practice was different from that for clinical trials. It seems that IC was not regarded as a subject of observational study, but the discussion on IC mainly concerned the disclosure of diagnosis to cancer patient in the 1980s[2].

In the 1990s, some Japanese IC studies were retrieved by our research. With the adoption of ICH-GCP, the Pharmaceutical Affairs Law and the new GCP ordinance for NDA trials were fully enforced in 1988 and training courses for CRC organized by the Ministry of Welfare were started[3]. Since then, medical institutions that conduct NDA trials have set up clinical trial management centers and institutional review boards (IRBs)*, and organize systems that include personnel. There was probably an increased awareness of the importance of protecting the rights and well-being of trial subjects since the enforcement of ICH-GCP and the cooperation of CRC in clinical trials. However, it was probably not until written consent became a legal obligation for pharmaceutical developers and medical institutions that research on IC for clinical trials was started in full swing.

The Japanese articles began to increase in number greatly from 2002. In the 2000s, the Declaration of Helsinki was amended several times[21], and some Japanese guidelines on medical research were also revised by ministries, agencies and medical societies[22,23]. As mentioned in the Declaration of Helsinki, each potential subject should be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail, as well as any other relevant aspects of the study[21]. During these changes surrounding clinical trials, several medical institutions built up their own infrastructures for clinical trials, and they accumulated and reported their experiences. Hence, the Japanese articles mainly focused on the contribution of CRC and the understanding of participants.

2) How should the quality of IC be evaluated?

From the viewpoint of IC quality, studies worldwide have mainly focused on participant understanding[25-27]. Our results also showed the same tendency in Japan[7,9,10,14,15,20]. This might have been influenced by the Declaration of Helsinki[21]. However, the extent of participant understanding is not always related to the process of IC or IC itself. The IC process is an individual issue and a subjective decision-making process, and contains psychosocial aspects such as medical culture and physician-patient relationship. Therefore it is difficult to identify all the factors associated with IC. In order to evaluate the process of IC in clinical trials, qualitative approaches would be useful, which include observation, interviews or verbal

*IRBs : To protect the rights of participants, IRBs have been set up in several hospitals in Japan since early in 1980s[24]. After adoption of ICH-GCP, the Pharmaceutical Affairs Law and the new GCP ordinance[3] empowered the function of IRBs.

interactions and focus on the meanings and interpretations of the participants[28]. To illustrate this approach, a representative article found by our comprehensive search is described below.

To reveal how CRC approached participants during IC, Nakamura et al.[12] took the verbatim records of IC communication between patients and CRCs. They conducted content analysis with the verbatim records, and categorized the approaches into 8 patterns including 'setting a good IC environment for participants and CRC,' 'providing information about the clinical trial' and 'estimating the stance of participants'. This is a pioneering IC study with a qualitative method in Japan.

The qualitative approach, which is not yet popular in IC evaluation, could reveal how gaps are formed between participants and medical professionals during the IC process. Therefore, to evaluate IC for clinical trials, we have to start to observe the process of IC in a multifaceted manner, namely, quantitatively and qualitatively.

3) Limitations of this study

Although we conducted a thorough search by comprehensive retrieval via electronic databases in order to identify articles on IC in clinical trials, our method has several limitations : 1) A database does not necessarily provide all the target articles that we would expect given the search conditions. Indeed, several articles dealing with IC for clinical trials in Japan were missed in our comprehensive retrieval[29-32]. 2) We excluded reports that did not contain abstracts. Therefore significant conference proceedings could have been overlooked.

5. Conclusion

After the enforcement of the new GCP, observational studies on IC for clinical trials have been conducted in Japan. However, most of them applied a quantitative research approach, and qualitative analysis of IC for clinical trials is limited. In Japan, IC is not aimed exclusively for the protection of trial subjects, and it is necessary to investigate the IC process by both qualitative and quantitative approaches.

Acknowledgments

This study was partly supported by Health Labor Sciences Research Grant and Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science.

References

1) Shimoyama M. Informed Consent—historical evolution and recent trend—. *The Journal of Clinical Science.* 1997 ; **33** (4) : 389-97 (in Japanese).
2) Yanagida K. *Inspiring Informed Consent.* Tokyo : Chuohoki Publishers, 1996 (in Japanese).
3) Good Clinical Practice (Amended March 27, 1997). Ministry of Health and Welfare.
4) Okazawa K, Takano M. Test subjects' perception of clinical trials of drugs and pharmacist clinical research coordinators' responsibilities : a questionnaire survey. *J Jpn Assoc Rural Med.* 2007 ; **56** (1) : 22-8 (in Japanese).
5) Fukui T, Rahman M, Morita S, Sakamoto J. Informed consent in the Candesartan Antihypertensive Survival Evaluation in Japan (CASE-J) trial : a survey of collaborating physicians. *Hypertens Res.* 2006 ; **29** (7) : 471-4.
6) Tsuji T, Wakasugi Y, Kanaya A, Yamasaki M, Nishida T, Kikutake K, et al. Questionnaire survey of physicians to assess their appreciation of clinical research and evaluate CRC practice in Kyushu University Hospital. *Jpn J Pharm Health Care Sci.* 2006 ; **32** (2) 164-73 (in Japanese).
7) Asada R, Noda K, Sakiyama E, Morita E, Sato T, Nishio M, et al. Survey of participants to clinical trial in Fukuoka University Hospital : Relationship between the participant's understanding of informed consent and their feeling of unease for clinical trials. *Medical Bulletin of Fukuoka University.* 2006 ; **33** (1) : 25-9 (in Japanese).
8) Matsuda I, Ooiwa Y, Fujimura M, Yoshida I, Ito S, Tsujimoto G, et al. A questionnaire concerning pediatric health care administered to pediatric nursing managers. *Jpn J Dev Pharmacol Ther.* 2003 ; **16** (1) : 123-34 (in Japanese).
9) Ando Sachiko, Ando Shoko, Kato T. Level of subject understanding of information in a clinical trial. *J Jpn Soc Nurs Res.* 2003 ; **26** (4) : 99-108 (in Japanese).
10) Watanabe K, Hara N, Negishi H, Sonehara W, Watanabe Y. Current situation and issues of taking consent of IND trials in National Defense Medical College, according to questionnaire to participants and physicians. *Journal of the National Defense Medical College.* 2002 ; **27** (4) : 184-90 (in Japanese).
11) Terada A, Kajiyama M, Nakatani S, Kashiwaguma R, Tezuka K, Kobayashi S, et al. Questionnaire for investigator and subject on clinical research coordinator in St. Marianna University Hospital. *Jpn J Clin Pharmacol Ther.* 2002 ; **33** (5) : 219-26 (in Japanese).
12) Nakamura N, Sakamoto T, Obara I, Nakao M, Morishita N, Naito M, et al. The methods for explanation of NDA trials by CRCs. *Quality Nursing.* 2002 ; **8** (7) : 619-24 (in Japanese).
13) Tanaka R, Ogawa S, Iizuka Y, Oomura M, Hayase N, Matsubara K, et al. The utility of research nurse during the process of informed consent for IND trials. *Jpn J Nursing Arts.* 2002 ; **48** (7) : 856-60 (in Japanese).
14) Nakano S. Reality of informed consent for IND trials in Japan, results of questionnaire comparing the participants of double-blinded comparative study in Japan with physicians-in-charge of them. *Jpn Pharmacol Ther.* 1997 ; **25** (9) : 2223-47 (in Japanese).
15) Kitajima K, Nakamura Y, Yao S, Yanagida T, Nakamura J, Tsubakihara Y, et al. Inquiry of patients about informed consent in clinical experimentation—the case of recombinant human erythropoietin on renal anemia in predialysis patients—. *Medical Journal of Osaka Prefectural Hospital* 1995 ; **18** (1) : 63-6 (in Japanese).

16) Yanagawa H, Nishiya M, Miyamoto T, Shikishima M, Imura M, Irahara M, et al. Clinical trials for drug approval : a pilot study of the view of doctors at Tokushima University Hospital. *J Med Invest.* 2006 ; **53**(3-4) : 292-6.
17) Rahman M, Morita S, Fukui T, Sakamoto J. Physicians' reasons for not entering their patients in a randomized controlled trial in Japan. *Tohoku J Exp Med.* 2004 ; **203**(2) : 105-9.
18) Ishii M, Ohashi Y. Influence of an educational videotape on attitudes toward participating in cohort studies : results of a randomized controlled trial. *Nippon Koshu Eisei Zasshi.* 2007 ; **54**(7) : 419-26.
19) Arita E, Iioka T, Ujihara A, Omori R, Koshiba S, Kamata S, et al. Evaluation of program to develop communication skills for health care professionals—Role-playing exercises introduced for training in informed consent process in clinical trials—. *Jpn J Pharm Health Care Sci.* 2008 ; **34**(8) : 727-35 (in Japanese).
20) Nishiwaki H, Yasuda I, Hayashi H. A study on informed consent in clinical trial. *J Hokkaido Rural Med.* 2009 ; **41** : 29-33 (in Japanese).
21) The Declaration of Helsinki (2008). World Medical Association.
22) The Ethical Guidelines for Clinical Studies (Amended July 31, 2008). Ministry of Health, Labour and Welfare, Japan.
23) The Ethical Guidelines for Epidemiology Studies (Amended August 16, 2007). Ministry of Education, Culture, Sports, Science and Technology, and Ministry of Health, Labour and Welfare, Japan.
24) Good Clinical Practice (October 2, 1989). Ministry of Health and Welfare.
25) Sugarman J, McCrory DC, Hubal RC. Getting meaningful informed consent from older adults : a structured literature review of empirical research. *J Am Geriatr Soc.* 1998 ; **46**(4) : 517-24.
26) Joffe S, Cook EF, Cleary PD, Clark JW, Weeks JC. Quality of informed consent in cancer clinical trials : a cross-sectional survey. *Lancet.* 2001 ; **358**(9295) : 1772-7.
27) Flory J, Emanuel E. Interventions to improve research participants' understanding in informed consent for research : a systematic review. *JAMA.* 2004 ; **292**(13) : 1593-601.
28) Holloway I, Wheeler S. Ethical issues in qualitative nursing research. *Nurs Ethics.* 1995 ; **2**(3) : 223-32.
29) Inoue Y, Kamiya E, Tachibana I, Yamazaki E, Atagi S, Kawahara M. Role of nurses in offering informed consent to clinical trials for advanced lung cancer. *Jpn J Lung Canc.* 2000 ; **40**(7) : 719-23 (in Japanese).
30) Takishita S. Large scale clinical trials for hypertention in Japan : obstacles in performing the trials. *Ryukyu Med J.* 2001 ; **20**(4) : 167-71 (in Japanese).
31) Kamei H, Ando S, Kato T, Naruse Y, Ichino M, Nabeshima T, et al. The role of clinical research coordinators on the quality control in clinical trials. *Jpn J Clin Pharmacol Ther.* 2003 ; **34**(1) : 19-23 (in Japanese).
32) Itoh K, Sasaki Y, Fujii H, Ohtsu T, Wakita H, Igarashi T, et al. Patients in phase I trials of anti-cancer agents in Japan : motivation, comprehension and expectations. *Br J Cancer.* 1997 ; **76**(1) : 107-13.

International Journal of Cardiology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis

Kana Shimada [a], Yu Mikami [b], Toshinori Murayama [a], Masayuki Yokode [a], Masatoshi Fujita [b], Toru Kita [a], Chiharu Kishimoto [a,*]

[a] Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Japan
[b] Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, Japan

ARTICLE INFO

Article history:
Received 26 November 2009
Received in revised form 13 April 2010
Accepted 30 May 2010
Available online xxxx

Keywords:
Atherosclerosis
Exercise
Behavioral stress
Atherosclerotic plaques

ABSTRACT

Background: We assessed the hypothesis whether behavioral stress may affect the development of atherosclerosis and whether regular exercise training may influence the composition of atherosclerotic plaques in apolipoprotein (apo) E-deficient mice.
Methods: Atherosclerosis was induced in apo E-deficient mice fed a high fat diet. Exercise training (45 min swimming, 3 times/week) was conducted, and behavioral stress was provoked by glass marble-burying procedure. Mice were treated with marble-burying, marble-burying behavior plus swimming training, and swimming alone over 8 weeks.
Results: Exercise training decreased the atherosclerotic lesions, but marble-burying behavior increased the lesions. The plaques containing macrophage accumulation with intercellular adhesion molecule-1 (ICAM-1) expression associated with reduced collagen contents were induced in the mice treated with marble-burying. However, ICAM-1 expression was suppressed and collagen contents were reversed in the mice that received marble-burying behavior plus exercise training. In addition, exercise alone and concomitant exercise training reduced the superoxide production in aortic walls, shown by dihydroethidium staining, compared with that in mice with marble-burying behavior alone. There were no significant differences in the serum lipids profiles among the groups.
Conclusions: Behavioral stress increased the atherosclerotic lesions and induced the adhesion molecule expression with superoxide production on the lesions in apo E-deficient mice. Exercise training may stabilize plaque lesions induced by marble-burying behavior in this animal model.

1. Introduction

Various stresses, inducing psychological stress, are now recognized to be an important contributor to cardiovascular diseases. Both clinical and experimental evidence supports the hypothesis that behavioral stress is linked to hypertension, cardiac arrhythmias, and sudden, unexpected death [1–5]. However, the specific sites of action and mechanisms involved in the development of atherosclerosis are not well known. Inflammation, cytokine overproduction, oxidative stress, and free radicals may be considered to be key mechanisms for the development of atherosclerosis [6]. On the other hand, exercise is a deterrent of cardiovascular disease, and its antiatherogenetic effects have been described [7–9].

Defensive burying is a behavior that can be elicited in rodents in response to aversive stimuli. The term is used to describe the behavior when rats or mice bury a shock prod, noxious food or dead conspecifics under a layer of bedding material [10,11]. In recent years, many investigators have used the marble-burying assay as a tool for assessing anxiety-like behaviors in mice [12].

In the present study, using apolipoprotein E-deficient mice, we have provided evidence for the induction of intercellular adhesion molecule-1 expression on atherosclerotic plaques by defensive burying behavior and for the suppression of the expression by exercise with the analysis of tissue superoxide production.

2. Materials and methods

2.1. Experimental atherosclerosis

The apolipoprotein E (apo E)-deficient 129ola×C57BL/6 hybrid mice were generous gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6 mice to produce F_1 hybrids. The F_1 apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to the subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-hour light–dark cycle with free access to food and water.

* Corresponding author. Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: +81 75 751 3197; fax: +81 75 751 4281.
E-mail address: kkishi@kuhp.kyoto-u.ac.jp (C. Kishimoto).

0167-5273/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ijcard.2010.05.057

Please cite this article as: Shimada K, et al, Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.05.057

After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when the animals were switched to a high fat diet containing 20% fat and 0.3% cholesterol as previously described [13,14].

The experimental protocols were approved by the institutional ethics committee for animal experiments of Kyoto University.

2.2. Procedure of marble-burying (Fig. 1)

The groups of 3 to 6 mice were placed in 23×17×14 cm cages for 30 min. Thereafter, mice were placed individually in a 23×17×14 cm cage with 20 glass marbles, 1.5 cm in diameter. The glass marbles were placed in close contact in the middle of the cage on a 5 cm layer of sawdust. The ceiling was smooth with a few-holes so that the mice could not cling to the cage ceiling. The mice were left in the cage with marbles for 30 min. After this, the test was terminated by removing the mice and counting the number of marbles that were more than two thirds covered by sawdust. The test was done 24 times during 8 weeks. The average value was calculated (Fig. 1).

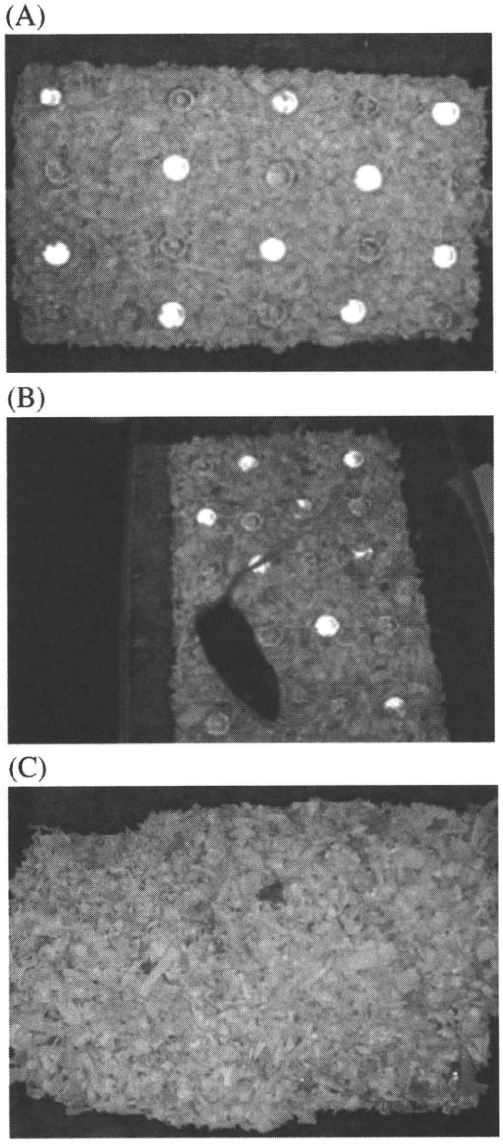

Fig. 1. Marble-burying behavior. (A)The 20 glass marbles were placed in a cage. (B) Mice were placed individually in a cage with for 30 min. After this, the test was terminated by removing the mice and counting the number of marbles that were covered by sawdust more than two thirds. (C) Number of marbles buried was calculated.

2.3. Experimental protocol

At 6 weeks of age, mice were divided into 4 groups and subjected to the study protocol. They were forced to marble-burying as mentioned before and to swim a hot water bath at 37 °C for 45 min per day 3 times on alternate days per week during 8 weeks as previously described [9]; control group ($n=14$), marble-burying group ($n=20$), marble-burying plus exercise group ($n=15$), and exercise group ($n=7$). It was already reported by us that this protocol of forced swimming exercise was not stressful for rodents [9]. The number of burying marbles was recorded at each time. The measurement was repeated three times at each time. At 14 weeks the mice were killed by puncture of the ventricle under ether anesthesia. The organs were weighed, and the ratios of organ weight to body weight were calculated.

2.4. Tissue processing

Mice were killed by bleeding with puncture of the ventricle. The vasculature was perfused with sterile phosphate buffered saline and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus 8 slides per mouse were prepared. All sections were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin [13,14]. Also, collagen contents were detected by Sirius red staining. The oil-red O and Sirius red stained sections were analyzed, blinded to group, as previously described [13–15].

2.5. Immunohistochemistry

Anti-macrophage (anti-Mϕ, M 3184, 1:400, PharMigen) and anti-intercellular adhesion molecule-1(ICAM-1) (M-19, 1:100, Santa Cruz Biotechnology) antibodies were applied to acetone-fixed cryosections of aortic roots. After being washed, the sections were then exposed to second antibodies (horseradish peroxidase-conjugated antibodies), and the horseradish peroxidase-conjugated antibody binding was visualized with diaminobenzidine. Sections were counterstained with methyl green or Mayer's hemotoxylin. Macrophages in the lesions were quantitatively evaluated as previously described [15,16]. Data were obtained by dividing the number of positively stained cells by all counterstained cells inside the internal elastic lamina. Three to five random microscopic fields were analyzed at ×200. ICAM-1 expression was qualitatively evaluated.

2.6. In situ defection of superoxide production

To evaluate in situ superoxide production from aorta, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [17–19]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10-μm thick sections, and incubated with 10 μM DHE at 37 °C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope. Superoxide production was demonstrated by red fluorescence labeling. For quantification of ethidium fluorescence from aortas, fluorescence (intensity × area) was measured using a high-power image. The validity of DHE staining for the evaluation of superoxide production from the atherosclerotic lesions was already reported by us [19].

2.7. Lipid measurement

Serum was separated by centrifugation and stored at −80 °C. Serum total cholesterol (TC) and triglyceride (TG) levels were measured with assay kits (Wako) according to the manufacturer's instructions.

2.8. Statistical analysis

Values were expressed as means ± SD. One-way ANOVA with subsequent Fisher protected least-significant difference tests was performed. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Organ weights (Table 1)

There were no significant differences in body weight, heart weight, or heart weight to body weight among the groups (Table 1).

3.2. Atherosclerotic lesions (Figs. 2 and 3)

The surface areas of aortic roots covered by fatty streak lesions were quantified in oil red-O-stained samples, and specimens among the groups were compared. Controls ($105.30\pm39.0\times10^3$ μm^2, $n=14$) and the marble-burying behavior group ($138.06\pm25.74\times10^3$ μm^2, $n=20$) developed extensive lesions in the root of the aorta (Fig. 2).

Please cite this article as: Shimada K, et al, Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.05.057

Table 1
Organ weights and lipid profiles.

	(n)	BW (g)	HW (g)	HW/BW (mg/g)	(n)	TC (mg/dl)	TG (mg/dl)
Control	14	31.5 ± 5.5	0.19 ± 0.04	6.03 ± 0.65	6	1307 ± 273	54.5 ± 28.4
Marble-burying	20	30.7 ± 6.2	0.21 ± 0.04	6.84 ± 0.63	6	1187 ± 283	68.0 ± 48.3
Marble-burying + Exercise	15	31.7 ± 4.9	0.20 ± 0.04	6.31 ± 0.60	6	1586 ± 304	52.5 ± 23.1
Exercise	7	31.7 ± 6.0	0.19 ± 0.05	5.99 ± 0.71	5	1233 ± 249	60.4 ± 34.4

(Mn ± SD).
BW = body weight, HW = heart weight, TC = total cholesterol, TG = triglyceride.

Exercise group ($61.62 \pm 15.60 \times 10^3$ μm², $n = 7$) showed the minimal lesions among the groups. The marble-burying behavior increased the severity of atherosclerosis significantly ($P<0.05$) compared with controls. In mice treated with marble-burying plus exercise ($69.42 \pm 19.50 \times 10^3$ μm², $n = 15$), the fractional area of lesions was reduced ($P<0.01$) compared with the marble-burying group as shown in Fig. 2.

Compared with the control ($38.22 \pm 10.14 \times 10^3$ μm²), collagen contents were decreased ($P<0.05$) by marble-burying behavior ($21.06 \pm 7.8 \times 10^3$ μm²) and were returned to the control levels by the treatment of marble-burying plus exercise ($39.78 \pm 17.94 \times 10^3$ μm²) (Fig. 3). In exercise group ($46.80 \pm 10.92 \times 10^3$ μm²), collagen contents were highest among the groups.

3.3. Inflammatory cell surface markers (Figs. 2 and 4)

The degree of macrophage-positive cells was decreased in the exercise-treated group and the marble-burying behavior-treated plus concomitant exercise-treated group compared with the other two groups (Fig. 2). The expression of ICAM-1 was increased by marble-burying behavior and was decreased by the concomitant exercise. The expression of ICAM-1 in exercise group was minimal (Fig. 4).

3.4. In situ superoxide production (Fig. 5)

To analyze the in situ superoxide production in the aortic wall, DHE staining was performed. Ethidium fluorescence was detected in the plaques. The intensity of DHE staining was increased by the marble-burying behavior, and was suppressed by the marble-burying plus swimming training group. The intensity of DHE staining in exercise group was lowest among the groups (Fig. 5).

3.5. Lipid profiles (Table 1)

There were no significant differences in the serum lipids profiles among the groups.

3.6. Numbers of marbles buried (Fig. 6)

Fig. 4 shows the numbers of marbles buried and the effects of exercise upon marble burying. The numbers in mice treated with exercise alone and concomitant exercise were significantly less compared with those in mice treated with marble-burying alone, which may reflect the less psychological stress-state in the exercise-treated group and the concomitant exercise-treated group (Fig. 6).

4. Discussion

In the current study, it was shown that (i) apo E-deficient mice fed a high fat diet over 8 weeks developed severe fatty streak lesions of aortic roots, (ii) behavioral stress increased the severity of atherosclerosis, and induced the atherosclerotic plaques with the increased ICAM-1 expression and superoxide production in aortic walls, and (iii) the propensity of the plaque unstability was reduced by the concomitant exercise treatment.

Chronic inflammation is thought to be of central importance in atherosclerosis [6,20]. It was shown that regular and chronic exercise could suppress overt and subclinical inflammation [21,22], based on the fact that atherosclerosis can be considered as generalized

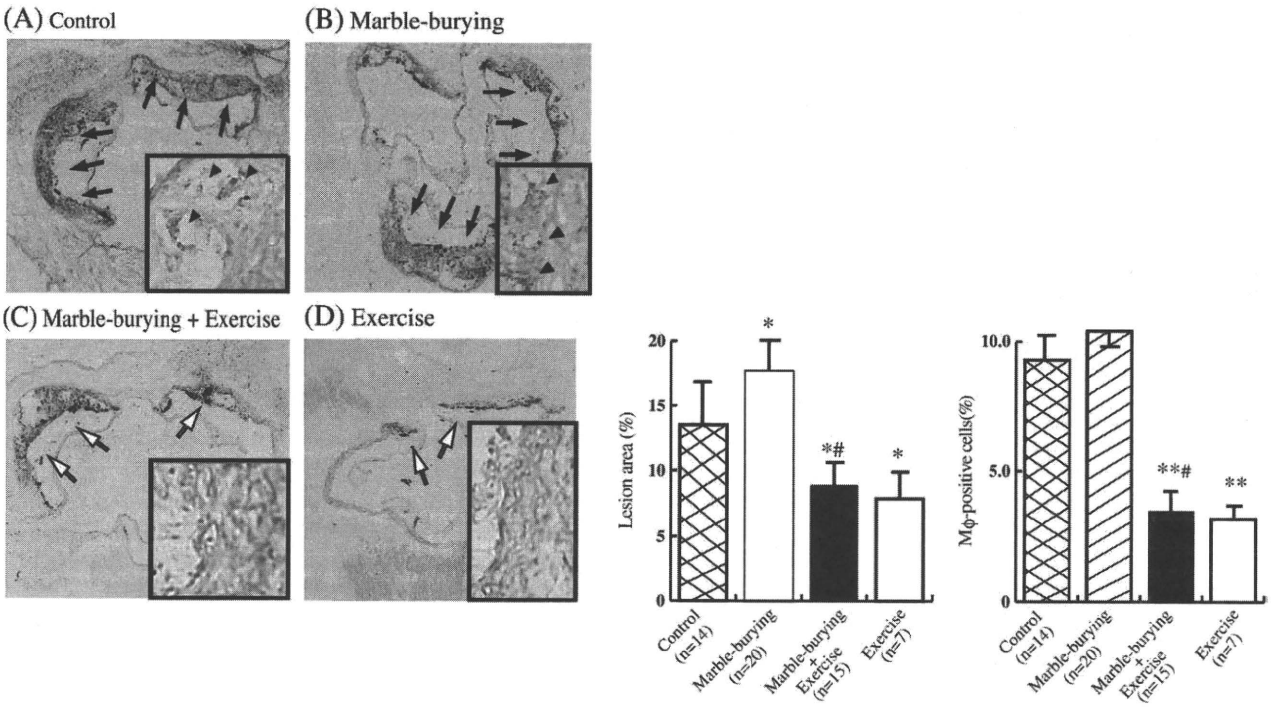

Fig. 2. Atherosclerotic lesions and, macrophage infiltration. The lesions (white arrows) in the marble-burying procedure plus exercise-treated mouse (C) and the exercise-treated mouse (D) were smaller and covered less of the inner circumference of the aortic root than those (black arrows) of the control mouse (A) and marble-burying procedure-treated mouse (B). Insets boxes show magnified samples for macrophages (Mϕ) (arrow-heads). Oil-red-O stain (A, B, C, D × 60). *$P<0.05$, **$P<0.01$ vs control group. #$P<0.01$ vs marble-burying group.

Please cite this article as: Shimada K, et al, Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.05.057

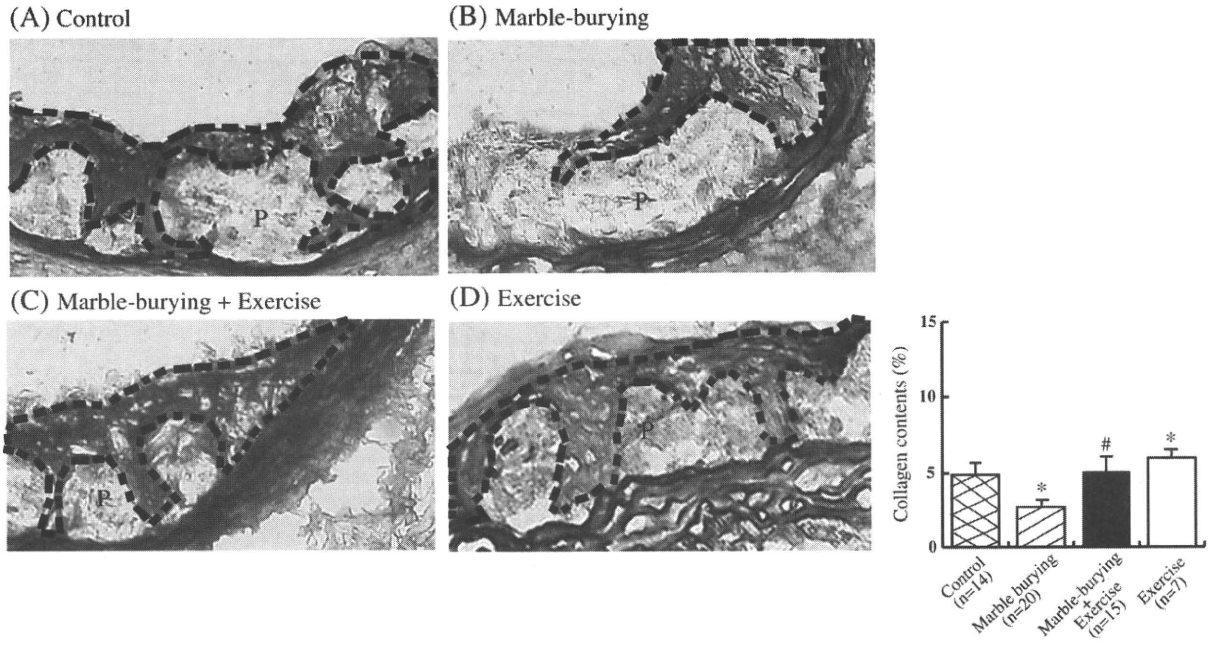

Fig. 3. Collagen contents. Compared with the control (A), collagen contents (red area, surrounded by dashed line) were decreased by marble-burying behavior (B), and were returned to the control levels by the treatment of marble-burying plus exercise (C). In exercise group (D), collagen contents were highest. P indicates plaques. Sirius red stain (A, B, C, D × 120). *$P<0.05$, vs control group. #$P<0.01$ vs marble-burying group.

manifestations of an inflammatory disease [6]. We and other investigators had already reported that experimental atherosclerosis in apo E-deficient mice was markedly suppressed by Fcγ portion of immunoglobulin administration, possibly by an antiinflammatory action via inhibitory Fcγ receptor IIB [15,16,23].

As mentioned previously, the so-called negative emotions, such as depression and anxiety, have been associated with the development of atherosclerosis and coronary artery disease [1–5]. Also, psychosocial distress and anxiety disorders are a significant risk factor for atherosclerosis [24–26]. Although the exact mechanisms are not yet clear, burying behavior in the mouse is provoked by glass marble, which is considered to be a kind of defensive burying [10–12].

In the present study, using the animal model of behavior stress induced by glass-burying procedure, we clearly demonstrated that behavioral stress increased the expression of adhesion molecule and superoxide production on atherosclerotic plaques, and that chronic and regular swimming training suppressed the expression of ICAM-1 and the overload of superoxide in aortic walls. It has already been established that the intensity of exercise training used in this study ameliorated the development of atherosclerosis in apo E-deficient mice [9], and that the degree of tissue DHE staining correlates with the superoxide production [17–19]. Thus, the decrease of the intensity of ethidium fluorescence expression in aortic walls may reflect the decrease of superoxide by exercise treatment.

The mechanisms by which exercise might benefit cardiovascular diseases are still unknown [7,8]. It is reported that exercise suppresses inflammation, hypertension, and atherosclerosis [7,8], and may reverse some cardiomyopathic conditions [27,28]. We already reported that chronic and regular exercise reduced the experimental atherosclerosis by the antioxidant effects [9]. Indeed, in the current study, exercise reduced the lesions and might stabilize atherosclerotic plaques by reducing the expression of macrophages and ICAM-1 as

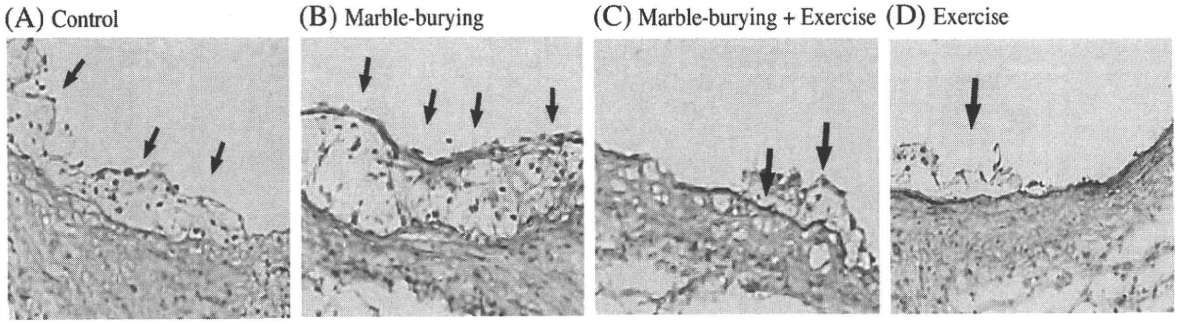

Fig. 4. Intercellular adhesion molecule-1 (ICAM-1) expression. The expression of ICAM-1 in the lesion (arrows) of the marble-burying plus exercise-treated mouse (C) were decreased compared with those of the control mouse (A) and marble-burying procedure-treated mouse (B). The expression of ICAM-1 in exercise group was minimal (D). ICAM-1 stain (A, B, C, D × 100)

Please cite this article as: Shimada K, et al, Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.05.057

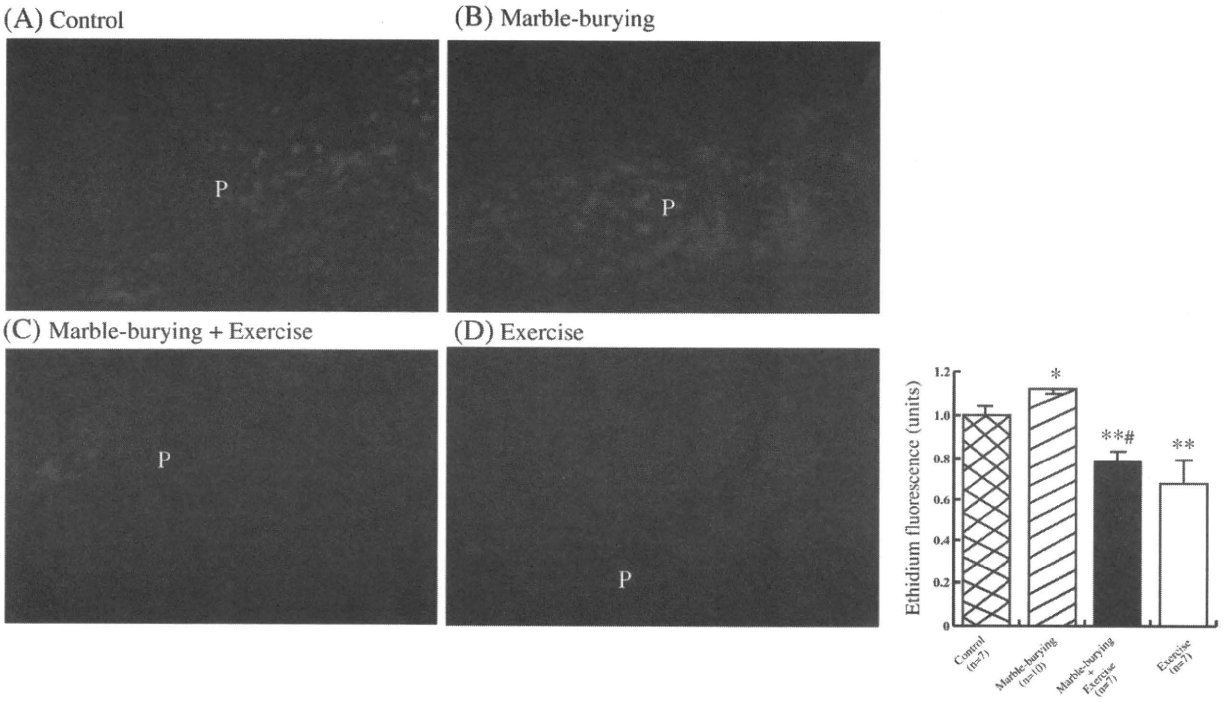

Fig. 5. Dihydroethidium (DHE) staining. Ethidium fluorescence was detected in the plaques (A). The intensity of DHE staining was increased by the marble-burying behavior (B), and was suppressed by the marble-burying plus swimming training group (C). The intensity of DHE staining in exercise group (D) was lowest among the group. P indicates plaques. DHE stain (A, B, C, D × 120). *$P<0.05$, **$P<0.01$ vs control group. #$P<0.01$ vs marble-burying group.

well as decreased intensity of DHE. Accordingly, regular exercise might be recommended for the clinical therapy for atherosclerotic patients.

The present study also demonstrated that exposure to behavioral stress induces the overexpression of ICAM-1 and the overproduction of superoxide in the aortic walls with relatively less amount of collagen contents in apo E-deficient mice, although it did not increase the severity of atherosclerosis significantly. This phenomenon may be of significant clinical relevance to humans. However, it should be noted that behavioral stress has been shown to have different effects in other strains of mice [10,11].

Behavioral stress is now recognized to be an important contributor to cardiovascular diseases, as mentioned before [1–5]. From the current study, however, appropriate and regular exercise may be recommended for the secondary prevention of atherosclerotic patients, especially having sustained psychological stress.

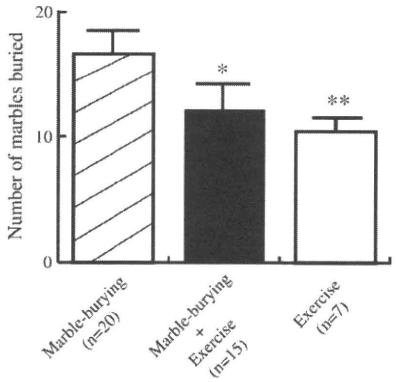

Fig. 6. Numbers of marbles buried. The numbers in mice treated with exercise alone and concomitant exercise were significantly less compared with those in mice treated with marble-burying alone. *$P<0.05$, **$P<0.01$ vs marble burying group.

Several limitations of the present study should be mentioned. First, the present study lacked the precise evaluation of hemodynamic study. Recently, it was reported that high heart rate and vigorous shear stress may affect the development of atherosclerosis [29]. However, it was already reported that the severity of atherosclerosis was reduced by the appropriate exercise protocol [9,30]. Second, the present study did not demonstrate the precise and molecular mechanisms why exercise might reduce the severity of experimental atherosclerosis. Third, the present experimental findings cannot be applied to the clinical settings directly because of the species differences of sensitivity for stress or anxiety.

In conclusion, behavioral stress may induce the overexpression of ICAM-1 and superoxide overproduction in the lesions in apo E-deficient mice, and concomitant exercise training may downregulate ICAM-1 expression and superoxide production, and reversed the collagen contents, resulting in the reduction of atherosclerosis. In view of the propensity of atherosclerotic plaques having adhesion molecule overexpression and superoxide overproduction, it may be concluded that exercise training may be useful for stabilizing the atherosclerotic plaques in clinical settings. Exploration of clinical usefulness of the results might be warranted.

Acknowledgement

Supported in part by research grants from The Shimizu Foundation for the Promotion of Immunology Research, The Universe Foundation, Chiyoda Kenko Organization, The Nakatomi Foundation, and Japanese Ministry of Education, Science, and Culture (18596772). The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [31].

Please cite this article as: Shimada K, et al, Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.05.057

References

[1] Schwartz PJ, LaRovere MT, Vanoli E. Autonomic nervous system and sudden cardiac death: experimental basis and clinical observations for post-myocardial infarction risk stratification. Circulation 1992;85(Suppl I):177–91.
[2] Specchia G, DeServi S, Falcone C, et al. Mental arithmetic stress testing in patients with coronary artery disease. Am Heart J 1984;108:56–63.
[3] Kamarck T, Jennings JR. Biobehavioral factors in sudden cardiac death. Psychol Bull 1991;109:42–75.
[4] Chauhan A, Mullins PA, Taylor G, Petch MC, Schofield PM. Effect of hyperventilation and mental stress on coronary blood flow in syndrome X. Br Heart J 1993;69:516–24.
[5] Lacy CR, Contrada RJ, Robbins ML, et al. Coronary vasoconstriction induced by mental stress. Am J Cardiol 1995;75:503–5.
[6] Ross R. Atherosclerosis: an inflammatory disease. N Engl J Med 1999;340:115–26.
[7] Koller A, Shephard RJ, Balady GJ. Exercise as cardiovascular therapy. Circulation 1999;99:963–72.
[8] Pynn M, Schäfer K, Konstantinides S, Halle M. Exercise training reduces neointimal growth and stabilizes vascular lesions developing after injury in apolipoprotein E-deficient mice. Circulation 2004;109:386–92.
[9] Okabe T, Shimada K, Hattori M, et al. Swimming reduces the severity of atherosclerosis in apolipoprotein E-deficient mice by antioxidant effects. Cardiovasc Res 2007;74:537–45.
[10] Broekkamp CL, Rijk HW, Joly-Gelouin D, Lloyd KL. Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. Eur J Pharmacol 1986;126:223–9.
[11] Pinel JP, Gorzalka BB, Ladak F. Cadaverine and putrescine initiate the burial of dead conspecifics by rats. Physiol Behav 1981;27:819–24.
[12] Thomas A, Burant A, Bui N, Graham D, Yuva-Payer L, Paylor R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology 2009;204:361–73.
[13] Murayama T, Yokode M, Kataoka H, et al. Intraperitoneal administration of anti-c-*fms* monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein E-deficient mice. Circulation 1999;99:1740–6.
[14] Okabe T, Kishimoto C, Shimada K, Murayama T, Yokode M, Kita T. Effects of MCI-186 (Edaravone), a novel free radical scavenger, upon experimental atherosclerosis in apolipoprotein E-deficient mice. Circ J 2006;70:1216–9.
[15] Yuan Z, Kishimoto C, Sano H, Shioji K, Xu Y, Yokode M. Immunoglobulin treatment suppresses atherosclerosis in apolipoprotein E-deficient mice via the Fc portion. Am J Physiol 2003;285:H899–906.
[16] Shioji K, Kishimoto C, Sasayama S. Fc receptor-mediated inhibitory effect of immunoglobulin therapy on autoimmune giant cell myocarditis: concomitant suppression of the expression of dendritic cells. Circ Res 2001;89:540–6.
[17] Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998;82:1298–305.
[18] Takaya T, Kawashima S, Shinohara M, et al. Angiotensin II type 1 receptor blocker telmisartan suppresses superoxide production and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice. Atherosclerosis 2006;186:402–10.
[19] Shimada K, Murayama T, Yokode M, et al. N-acetylcysteine reduces the severity of atheroslclerosis in apolipoprotein E-deficient mice by reducing superoxide production. Circulation 2009;73:1337–41.
[20] Kondo N, Nomura M, Nakaya Y, Ito S, Ohguro T. Association of inflammatory marker and highly sensitive C-reactive protein with aerobic exercise capacity, maximum oxygen uptake and insulin resistance in healthy middle-aged volunteers. Circ J 2005;69:452–7.
[21] Gielen S, Adams V, Möbius-Winkler S, et al. Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure. J Am Coll Cardiol 2003;42:861–8.
[22] Milani RV, Lavie CJ, Mehra MR. Reduction in C-reactive protein through cardiac rehabilitation and exercise training. J Am Coll Cardiol 2004;43:1056–61.
[23] Nicoletti A, Kaveri S, Caligiuri G, Bariéty J, Hansson GK. Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. J Clin Invest 1998;102:910–8.
[24] Paterniti S, Zureik M, Ducimetière P, Touboul PJ, Fève JM, Alpérovitch A. Sustained anxiety and 4-year progression of carotid atherosclerosis. Arterioscler Thromb Vasc Biol 2001;21:136–41.
[25] Todaro JF, Shen BJ, Niaura R, Spiro 3rd A, Ward KD. Effects of negative emotions on frequency of coronary heart disease. The Normative Aging study. Am J Cardiol 2003;92:901–6.
[26] Lavie CJ, Milani RV. Prevalence of anxiety in coronary patients with improvement following cardiac rehabilitation and exercise training. Am J Cardiol 2004;93:336–9.
[27] Maloyan A, Gulick J, Glabe CG, Kayed R, Robbins J. Exercise reverses preamyloid oligomer and prolongs survival in αB-crystallin-based desmin-related cardiomyopathy. Proc Natl Acad Sci USA 2007;104:5995–6000.
[28] Konhilas JP, Watson PA, Maass A, et al. Exercise can prevent and reverse the severity of hypertrophic cardiomyopathy. Circ Res 2006;98:540–8.
[29] Giannoglou G, Chatzizisis YS, Zamboulis C, Parcharidis GE, Mikhailidis DP, Louridas GE. Elevated heart rate and atherosclerosis: an overview of the pathogenetic mechanisms. Int J Cardiol 2008;126:302–12.
[30] Matsumoto Y, Adams V, Jacob S, Mangner N, Schuler G, Linke A. Regular exercise training prevents aortic valve disease in low-density lipoprotein-receptor-deficient mice. Circulation 2010;121:759–67.
[31] Coats AJ. Ethical authorship and publishing. Int J Cardiol 2009;131:149–50.

Please cite this article as: Shimada K, et al, Atherosclerotic plaques induced by marble-burying behavior are stabilized by exercise training in experimental atherosclerosis, Int J Cardiol (2010), doi:10.1016/j.ijcard.2010.05.057

ARTICLE IN PRESS
+ MODEL
Nutrition, Metabolism & Cardiovascular Diseases (2010) xx, 1–7

available at www.sciencedirect.com
ScienceDirect
journal homepage: www.elsevier.com/locate/nmcd

Nutrition, Metabolism & Cardiovascular Diseases

Olmesartan, a novel angiotensin II type 1 receptor antagonist, reduces severity of atherosclerosis in apolipoprotein E deficient mice associated with reducing superoxide production ☆

K. Shimada [a], T. Murayama [a], M. Yokode [a], T. Kita [a], M. Fujita [b], C. Kishimoto [a,*]

[a] *Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, 54 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

Received 21 October 2009; received in revised form 10 December 2009; accepted 31 December 2009

KEYWORDS
Atherosclerosis;
Angiotensin II type 1 receptor antagonist;
Oxidative stress;
Cytokines

Abstract *Background and Aim:* Oxidative stress may play an important role in the development of atherosclerosis. Some angiotensin II type 1 (AT_1) receptor antagonists have the capacity of reducing oxidative stress in addition to the hemodynamic actions. Accordingly, we assessed the hypothesis that olmesartan, a novel AT_1 receptor antagonist, reduced the severity of atherosclerosis in apolipoprotein (apo) E-deficient mice associated with reducing oxidative stress.

Methods and results: Atherosclerosis was induced in apo E-deficient mice fed a high fat diet. Mice were intraperitoneally treated with an injection of olmesartan (1 mg/kg/day) daily over 8 weeks, and were compared with the untreated controls. Blood pressure was not changed significantly by the olmesartan treatment. Fatty streak plaque developed in apo E-deficient mice, and was suppressed in mice that received olmesartan. In addition, olmesartan reduced not only superoxide production but the overload of oxidative stress in aortic walls. There were no significant differences in serum lipid levels between olmesartan-treated and -untreated groups. In vitro study showed that both olmesartan and its active metabolite RNH-6270, an enantiomer of olmesartan, suppressed interferon-γ, macrophage inflammatory protein-2, and thioredoxin (a marker of oxidative stress) concentrations in cultured cells.

☆ Supported in part by research grants from The Univers Foundation, Chiyoda Kenko Organization, The Nakatomi Foundation, and Japanese Ministry of Education, Science, and Culture (18596772).
* Corresponding author. Tel.: +81 (075) 751 3197; fax: +81 (075) 751 4281.
E-mail address: kkishi@kuhp.kyoto-u.ac.jp (C. Kishimoto).

0939-4753/$ - see front matter
doi:10.1016/j.numecd.2009.12.016

Please cite this article in press as: Shimada K, et al., Olmesartan, a novel angiotensin II type 1 receptor antagonist, reduces severity of atherosclerosis in apolipoprotein E deficient mice associated with reducing superoxide production, Nutr Metab Cardiovasc Dis (2010), doi:10.1016/j.numecd.2009.12.016

Conclusion: Olmesartan may suppress atherosclerosis via reducing not only superoxide production but also the overload of oxidative stress in this animal model.

Introduction

Inflammation, cytokine overproduction, oxidative stress, and free radicals may be considered to be key mechanisms for the development of atherosclerosis [1]. In addition, the significance of renin-angiotensin system in the development of atherosclerosis is now well known [2]. Angiotensin II is a major mediator of oxidative stress by activating NADH/NAD(P)H oxidase via the type 1 receptor, which results in the production of the superoxide anion. Accordingly, angiotensin II has deleterious effects on vessel walls.

Recent reports indicate that some angiotensin type 1 (AT_1) receptor antagonists inhibit inflammatory reactions in macrophages and myocardium [3–5]. AT_1 receptor antagonists have also been reported to inhibit interleukin (IL)-1 production as well as free radical production [6]. These results imply that olmesartan, a novel AT_1 receptor antagonist, may be an effective agent in countering inflammatory reactions and oxidative stress of vessel walls [5–7]. In addition, it was recently reported that olmesartan suppressed the development of atherosclerosis by various effects, such as prevention of endothelial disruption, decreasing effects of macrophage accumulation in the lesions, and so on [8–13]. However, the exact mechanism of olmesartan on atherosclerosis are still unknown.

In the present study, using apolipoprotein E-deficient mice which is a well-known animal model of experimental atherosclerosis [14–16], we have provided evidence for the lowering effects of atherosclerotic lesions by olmesartan, focusing upon inhibitory effects for free radical and oxidative stress production in vivo and in vitro.

Methods

Experimental atherosclerosis

The apolipoprotein E (apo E)-deficient 129ola × C57BL/6 hybrid mice were generous gifts of Dr. Edward M. Rubin (University of California, Berkeley, CA). These mice were mated with C57BL/6 mice to produce F_1 hybrids. The F_1 apo $E^{+/-}$ mice were then backcrossed to C57BL/6 mice for 10 generations. Mice homogeneous for the apo E-null allele on a C57BL/6 background were subsequently generated. Male mice were subjected to the subsequent experiments. The mice were kept in a temperature-controlled facility on a 14:10-h light-dark cycle with free access to food and water.

After being weaned at 4 weeks of age, mice were fed a normal chow diet (Oriental Yeast) until 6 weeks of age, when the animals were switched to a high fat diet containing 20% fat and 0.3% cholesterol as previously described [17,18].

The experimental protocols were approved by the institutional ethics committee for animal experiments of Kyoto University.

Treatment protocol

At 6 weeks of age, mice were treated daily with an intraperitoneal injection of either saline (olmesartan-untreated group, $n = 9$) or 1 mg/kg/day of olmesartan (olmesartan-treated group, $n = 9$) for 8 weeks. The dosage of the drug without affecting the blood pressure significantly was determined from the previous report [5]. Blood pressure and heart rate were periodically determined by the tail-cuff method using a photoelectric tail-cuff detection system (model BP-98A, Softron, Tokyo, Japan). The measurement was repeated three times at each time. At 14 weeks the mice were sacrificed by puncture of the ventricle under ether anesthesia. The organs were weighed, and the ratios of organ weight to body weight were calculated. Olmesartan and its active metabolite RNH-6270 (an enantiomer of olmesartan) [19] were kindly provided by Daiichi-Sankyo Company (Tokyo, Japan).

Tissue processing

Mice were sacrificed by bleeding with the puncture of the ventricle. The vasculature was perfused with sterile phosphate buffered saline (PBS) and 6.8% sucrose. The root of the aorta was dissected under a microscope and frozen in OCT embedding medium for serial cryosectioning covering 1.0 mm of the root. The first section was harvested when the first cusp became visible in the lumen of the aorta. Four sections of 6 μm thickness were harvested per slide, and thus eight slides per mouse were prepared. All sections were immersed for 15 s in 60% isopropanol, stained for 30 min in a saturated oil-red-O solution at room temperature, counterstained with hematoxylin, and then mounted under coverslips with glycerol gelatin [18].

Quantitation of atherosclerotic lesions

The oil-red-O-stained sections were analyzed at a magnification of ×10, as previously described [17,18]. The image was captured directly from the RGB camera attached to a light microscope and displayed on a microcomputer to quantify the cross-sectional surface area of the lesion. The fractional area of the lesion was calculated by dividing the whole vessel area including the lumen, intima, media, and adventitia, as previously described [17,18]. For each animal, 20 sections, i.e., every fourth section, were examined, and the mean of the fraction area was calculated and expressed as a percentage.

In situ defection of superoxide production

To evaluate in situ superoxide production from vessel walls, unfixed frozen cross sections of the specimens were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [7,20,21]. In the

Please cite this article in press as: Shimada K, et al., Olmesartan, a novel angiotensin II type 1 receptor antagonist, reduces severity of atherosclerosis in apolipoprotein E deficient mice associated with reducing superoxide production, Nutr Metab Cardiovasc Dis (2010), doi:10.1016/j.numecd.2009.12.016