40. Coxon JP, Oades GM, Kirby RS, Colston KW. Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation. BJU Int 2004;94(1):164–170.
41. Marten A, Lilienfeld-Toal M, Buchler MW, Schmidt J. Zoledronic acid has direct antiproliferative and antimetastatic effect on pancreatic carcinoma cells and acts as an antigen for delta2 gamma/delta T cells. J Immunother 2007;30(4):370–377.
42. Sato K, Kimura S, Segawa H, Yokota A, Matsumoto S, Kuroda J, Nogawa M, Yuasa T, Kiyono Y, Wada H, Maekawa T. Cytotoxic effects of gammadelta T cells expanded ex vivo by a third generation bisphosphonate for cancer immunotherapy. Int J Cancer 2005;116(1):94–99.
43. Roelofs AJ, Jauhiainen M, Monkkonen H, Rogers MJ, Monkkonen J, Thompson K. Peripheral blood monocytes are responsible for gamma delta T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. Br J Haematol 2009;144(2):245–250.
44. Reid IR, Brown JP, Burckhardt P, Horowitz Z, Richardson P, Trechsel U, Widmer A, Devogelaer JP, Kaufman JM, Jaeger P, Body JJ, Brandi ML, Broell J, Di Micco R, Genazzani AR, Felsenberg D, Happ J, Hooper MJ, Ittner J, Leb G, Mallmin H, Murray T, Ortolani S, Rubinacci A, Saaf M, Samsioe G, Verbruggen L, Meunier PJ. Intravenous zoledronic acid in postmenopausal women with low bone mineral density. N Engl J Med 2002;346(9):653–661.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Differentiation Capacity of Hepatic Stem/Progenitor Cells Isolated From D-Galactosamine-Treated Rat Livers

Norihisa Ichinohe,[1] Naoki Tanimizu,[1] Hidekazu Ooe,[1] Yukio Nakamura,[1,2] Toru Mizuguchi,[2] Junko Kon,[1] Koichi Hirata,[2] and Toshihiro Mitaka[1]

Oval cells and small hepatocytes (SHs) are known to be hepatic stem and progenitor cells. Although oval cells are believed to differentiate into mature hepatocytes (MHs) through SHs, the details of their differentiation process are not well understood. Furthermore, it is not certain whether the induced cells possess fully mature functions as MHs. In the present experiment, we used Thy1 and CD44 to isolate oval and progenitor cells, respectively, from D-galactosamine-treated rat livers. Epidermal growth factor, basic fibroblast growth factor, or hepatocyte growth factor could trigger the hepatocytic differentiation of sorted Thy1$^+$ cells to form epithelial cell colonies, and the combination of the factors stimulated the emergence and expansion of the colonies. Cells in the Thy1$^+$-derived colonies grew more slowly than those in the CD44$^+$-derived ones *in vitro* and *in vivo* and the degree of their hepatocytic differentiation increased with CD44 expression. Although the induced hepatocytes derived from Thy1$^+$ and CD44$^+$ cells showed similar morphology to MHs and formed organoids from the colonies similar to those from SHs, many hepatic differentiated functions of the induced hepatocytes were less well performed than those of mature SHs derived from the healthy liver. The gene expression of cytochrome P450 1A2, tryptophan 2,3-dioxygenase, and carbamoylphosphate synthetase I was lower in the induced hepatocytes than in mature SHs. In addition, the protein expression of CCAAT/enhancer-binding protein alpha and bile canalicular formation could not reach the levels of production of mature SHs. *Conclusion*: The results suggest that, although Thy1$^+$ and CD44$^+$ cells are able to differentiate into hepatocytes, the degree of maturation of the induced hepatocytes may not be equal to that of healthy resident hepatocytes. (HEPATOLOGY 2012; 57:1192-1202)

The liver normally exhibits a very low level of cell turnover, but when loss of mature hepatocytes (MHs) occurs, a rapid regenerative response is elicited from all cell types in the liver to restore the organ to its initial state. The loss may occur as a result of toxic injury, viral infection, trauma, or surgical resection. Because hepatocytes are the major functional cells of the liver, large-scale hepatocytic loss becomes a trigger for regeneration, and replication of existing hepatocytes is generally the quickest, most efficient way to compensate for the lost functions. However, when the replication of hepatocytes is delayed or entirely inhibited, hepatic stem/progenitor cells (HPCs) are activated.[1-3] As HPCs, oval cells and small hepatocytes (SHs) are well known. Oval cells were first reported to be cells that possessed an ovoid nucleus and

Abbreviations: Abs, antibodies; AFP, alpha-fetoprotein; Alb, albumin; BC, bile canaliculus; bFGF, basic fibroblast growth factor; BrdU, 5-bromo-2'-deoxyuridine; BW, body weight; CPS-I, carbamoylphosphate synthetase I; CK, cytokeratin; C/EBP, CCAAT/enhancer-binding protein; CYP1A2, cytochrome P450 1A2; 3D, three-dimensional; DPPIV, dipeptidylpeptidase IV; ECM, extracellular matrix; EGF, epidermal growth factor; FD, fluorescent diacetate; FGF, fibroblast growth factor; GalN, D-galactosamine; HA, hyaluronic acid; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; HPCs, hepatic stem/progenitor cells; ICC, immunocytochemistry; INF-γ, interferon-gamma; IP, intraperitoneally; LI, labeling index; MH, mature hepatocyte; mRNA, messenger RNA; PBS, phosphate-buffered saline; PH, partial hepatectomy; qPCR, quantitative polymerase chain reaction; RET, retrorsine; SH, small hepatocyte; TAT, tyrosine aminotransferase; TGF, transforming growth factor; TNF-α, tumor necrosis factor alpha; TDO, tryptophan 2,3-dioxygenase;

From the [1]Department of Tissue Development and Regeneration, the Research Institute for Frontier Medicine, and [2]First Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan.

Received January 31, 2012; accepted September 8, 2012.

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, Grant-in-Aid for Scientific Research (C) (19566021; to N.I.), Grants-in-Aid for Young Scientists (B) (22790385, to N.I.; and 19790294, to J.K.), a grant from the Yuasa Memorial Foundation (to T.M.), and Grants-in-Aid for Scientific Research (B) (22390259, to K.H.; and 21390365, to T.M.), a program for developing the supporting system for upgrading the education and research (to T.M.).

Dr. Kon is currently affiliated with Gene Techno Science Co. Ltd., Sapporo, Japan.

scant cytoplasm.[4] The appearance of oval cells has been reported in rat livers treated with hepatotoxins, such as 2-acetylaminofluorene (2-AAF), combined with partial hepatectomy (PH) and D-galactosamine (GalN).[1,5–7] In GalN-induced rat liver injury, it has been shown that oval cells appear in the periportal area and differentiate into MHs through basophilic small-sized ones.[8,9] Oval cells show a wide range of phenotypic heterogeneity, and cytokeratins (CKs) 7 and 19, alpha-fetoprotein (AFP), CD34, c-kit, and Thy1 have been reported as markers for them.[1,2,5–7]

On the other hand, SHs are a subpopulation of hepatocytes, and cells isolated from healthy adult rats[10,11] and human livers[12] can clonally proliferate to form colonies and differentiate into MHs *in vitro*.[11,13] Recently, we identified CD44 as a specific marker of SHs.[14] In GalN-treated rat livers, $CD44^+$ cells appear near the periportal area between $Thy1^+$ oval cells and resident hepatocytes soon after the emergence of $Thy1^+$ oval cells.[15] In addition, we previously showed that $Thy1^+$ oval cells differentiate into hepatocytes through $CD44^+$ cells.[15,16] Our data suggested that cells sequentially converted from $Thy1^+CD44^-$ to $Thy1^+CD44^+$ and then to $Thy1^-CD44^+$ cells during the process of hepatocytic differentiation of oval cells.[15,16] Furthermore, sorted $Thy1^+$ and $CD44^+$ cells could repopulate host livers when they were transplanted into rat livers treated with retrorsine (RET) and two-thirds PH.

Although most oval cells are thought to differentiate into MHs, the details of their differentiation process, such as factors for hepatic commitment, characteristics of intermediate cells, and their fates are not well understood. In addition, it has not been elucidated whether the induced hepatocytes differentiate to possess the same capabilities as MHs. In the present experiment, we aimed to clarify which factors might induce hepatocytic differentiation of $Thy1^+$ cells and to examine how $Thy1^+$ cells could differentiate into hepatocytes through $CD44^+$ cells. In addition, we examined whether the $Thy1^+$ and $CD44^+$ cells could differentiate into fully MHs, as with those in the healthy adult liver.

Materials and Methods

Animals and Liver Injury Model. Male F344 rats (dipeptidylpeptidase IV [DPPIV]$^+$ strain; Sankyo Lab Service Corporation, Inc., Tokyo, Japan), weighing 150-200 g, were used. All animals received humane care, and the experimental protocol was approved by the committee on laboratory animals according to Sapporo Medical University guidelines. For GalN-injured livers, GalN (75 mg/100 g body weight [BW] dissolved in phosphate-buffered saline [PBS]; Acros, Geel, Belgium) was intraperitoneally (IP) administered.[14] For the transplantation experiment, female F344 rats (DPPIV$^-$ strain; Charles River Laboratories, Wilmington, MA) were (IP) given two injections of RET (30 mg/kg BW; Sigma-Aldrich Chemical Co., St. Louis, MO), 2 weeks apart,[17] and 4 weeks after the second injection, two-thirds PH was performed (RET/PH liver). Sorted DPPIV$^+$ cells (5×10^5 cells/0.5 mL) were transplanted into RET/PH livers (DPPIV$^-$) through the spleen (at least 3 rats per group).

Isolation and Culture of Cells. Rats were used to isolate hepatic cells by the collagenase perfusion method, as previously described.[18] After perfusion, the cell suspension was centrifuged at $50 \times g$ for 1 minute. The supernatant and the precipitate were used for sorting $Thy1^+$ and $CD44^+$ cells and preparing MHs, respectively. The procedure used for cell sorting was as previously described,[15] with some modifications. Antibodies (Abs) used for cell sorting are listed in Supporting Table 1. $Thy1^+CD44^+$ cells were sorted from $CD44^+$ cell and $Thy1^+$ cell fractions by using anti-Thy1 or CD44 Abs, respectively, and both were pooled. Furthermore, $Thy1^+$ and $CD44^+$ cells were also separated from $CD44^-$ and $Thy1^-$ cell fractions, respectively. After the number of viable cells was counted, 1×10^5 viable cells were plated in 12-well plates (Corning Inc., Corning, NY) and cultured in the medium listed in Supporting Table 2. The medium was replaced with fresh medium thrice-weekly.

To examine whether cells in the colonies could fully differentiate into MHs and form functional bile canaliculi (BCs), $Thy1^+CD44^-$ and $Thy1^-CD44^+$ cells sorted from GalN-D3 and SHs derived from a healthy liver were cultured for 10 days. Thereafter, some dishes were treated with Matrigel (BD Biosciences, San Diego, CA) for 10 days. To enhance the organoid formation of the colonies, as previously reported,[19]

Address reprint requests to: Norihisa Ichinohe, D.D.S., Ph.D., Department of Tissue Development and Regeneration, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo 060-8556, Japan. E-mail: nichi@sapmed.ac.jp; fax: +81-11-615-3099.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.26084
Potential conflict of interest: Nothing to report.
Additional Supporting Information may be found in the online version of this article.

colonies were separated from dishes by using Cell Dissociation Solution (Sigma-Aldrich), and colonies (2×10^3) were replated on collagen-coated dishes. Cells were cultured in the induction medium (Supporting Table 2) for 14 days. Cloning rings were used to isolate total RNA of each colony. At least two separate experiments were performed, and more than five colonies were investigated.

GeneChip Analysis, RNA Isolation, and Real-Time Polymerase Chain Reaction. Details are shown in the Supplementary Methods.

Immunostaining. For detecting $CD44^+$ colonies, cells were fixed with cold absolute ethanol at 10 days after plating, and immunocytochemistry (ICC) for CD44 was carried out. Details of staining were previously reported.[15] The numbers of $CD44^+$ colonies at days 5 and 10 were counted, and positivity was calculated. Three separate experiments were performed. To measure the labeling index (LI), 40 μM of 5-bromo-2'-deoxyuridine (BrdU) were added to the medium 24 hours before fixation. In double ICC for CD44 and BrdU, a combination of the avidin-biotin peroxidase complex method (Vectastain ABC Elite Kit; Vector Laboratories Inc., Burlingame, CA) and the alkaline phosphatase method was used. For fluorescent immunohistochemistry, sliced liver samples were frozen using isopentane/liquid nitrogen, and materials were kept at −80°C until use. All Abs used for immunostaining are listed in Supporting Table 1. Sections were embedded with 90% glycerol including 0.01% *p*-phenylenediamine and 4,6-diamidino-2-phenylindole. A confocal laser microscope (Olympus, Tokyo, Japan) was used for observation, and findings were analyzed using DP Manager (Olympus).

Treatment With Fluorescein Diacetate. As previously reported,[20] fluorescein diacetate (FD; Sigma-Aldrich) was dissolved in dimethyl sulfoxide, and the solution was diluted with the culture medium. Then, 0.25% FD was added to the medium, and the dish was rinsed three times with warm PBS. Fluorescent images were immediately photographed using a phase-contrast microscope equipped with a fluorescence device (Olympus).

Enzyme Histochemisty for DPPIV. To identify donor cells, enzyme histochemistry for DPPIV was carried out. DPPIV enzyme activity was detected as previously described.[15] $DPPIV^+$ foci in livers were photographed using a microscope equipped with a CCD camera, and the area of each focus was measured using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

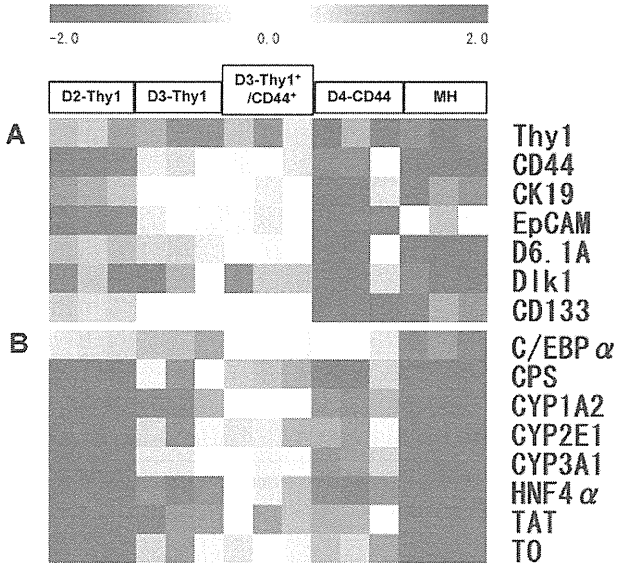


Fig. 1. Gene expression of sorted GalN-D2-$Thy1^+$ cells, D3-$Thy1^+$ cells, D3- $Thy1^+/CD44^+$ cells, and D4-$CD44^+$ cells. The gene-expression pattern of sorted cells was analyzed using GeneChip (Affymetrix, Inc., Santa Clara, CA). MHs isolated from a healthy adult rat liver were used as a control. A heatmap for genes that are classified into (A) stem cell and HPC markers and (B) hepatic markers. Relative expression of genes is shown in $\log_2$ scale. Increases in mRNA level are represented as shades of red and decreases as shades of blue.

Statistical Analysis. All data were analyzed using Turkey-Kramer's multiple comparison test. Level of statistical significance was $P < 0.05$. Experimental results are expressed as the geometric mean ± standard deviation.

Results

Characterization of Isolated Cells From Livers Treated by GalN. As previously reported,[15] $Thy1^+$ cells differentiated into hepatocytes through a $CD44^+$ intermediate state, as shown with clonally cultured $Thy1^+$ cells and cell transplantation. This transition likely happened in the GalN-treated rat liver as well. GeneChip data (Affymetrix, Inc., Santa Clara, CA) indicated that the immature hepatocyte markers, Dlk[21] and AFP were up-regulated in $Thy1^+CD44^+$ and $Thy1^-CD44^+$ cells, whereas markers related to hepatic differentiation were gradually up-regulated during the transition from Thy1-D2 to CD44-D4 cells (Fig. 1). The results also suggested that most D2-$Thy1^+$ cells were not committed to the hepatic lineage. This is consistent with our previous finding that $Thy1^+$ cells isolated from GalN-D2 could form a few epithelial cell colonies in the standard medium for SH induction, whereas those from GalN-D3 certainly formed colonies consisting of $CD44^+$ cells. Therefore, we

Table 1. Effects of Growth Factors and Cytokines on the Formation of Epithelial Cell Colonies

Growth Factors	Numbers of Colonies/Well	Numbers of Cells/Colony
Control	0	0
EGF	4.5 ± 3.8	41.4 ± 1.8
bFGF	0.3 ± 0.6	13.0 ± 0.0
HGF	1.0 ± 1.7	24.6 ± 11.7
LIF	0	0
TNF-α	0	0
IFN-γ	0	0
OSM	0	0
PDGF-BB	0	0
SCF	0	0
IL-6	0	0
TGF-β1	0	0
TGF-β2	0	0

Abbreviations: LIF, leukemia inhibitory factor; OSM, oncostatin M; PDGF-BB, plate-let-derived growth factor BB; SCF, stem cell factor.

considered the possibility that Thy1$^+$ cells became the hepatocyte lineage between D2 and D3. To specify the factors that trigger hepatic commitment, we compared expression patterns of genes related to receptors of growth factors and cytokines and selected 12 candidates (Table 1).

Induction of Epithelial Cell Colonies by Growth Factors. D2-Thy1$^+$ cells were cultured in the medium supplemented with each factor. To elucidate the formation of epithelial cell colonies, ICC for CD44 was conducted 10 days after plating. Of the 12 candidates, only epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) could induce colonies (Fig. 2A; Table 1). CD44 expression of cells varied among the colonies, and some colonies consisted of cells with low expression of CD44 (CD44$^-$ cells). Next, we examined whether bFGF and/or HGF could enhance the formation and expansion of colonies in the culture with EGF (Fig. 2B). Compared to EGF only (control), the addition of bFGF or HGF did not enhance the frequency of colony formation. In the combination of EGF and bFGF or HGF, the number of cells per colony increased to twice as many as in the control (Fig. 2C). In addition, the combination of the three factors also dose dependently increased the number of cells per colony. These results suggested that a certain number of Thy1$^+$ cells possessed the ability to differentiate into hepatic cells, and that the induction was initiated by EGF, bFGF, and/or HGF.

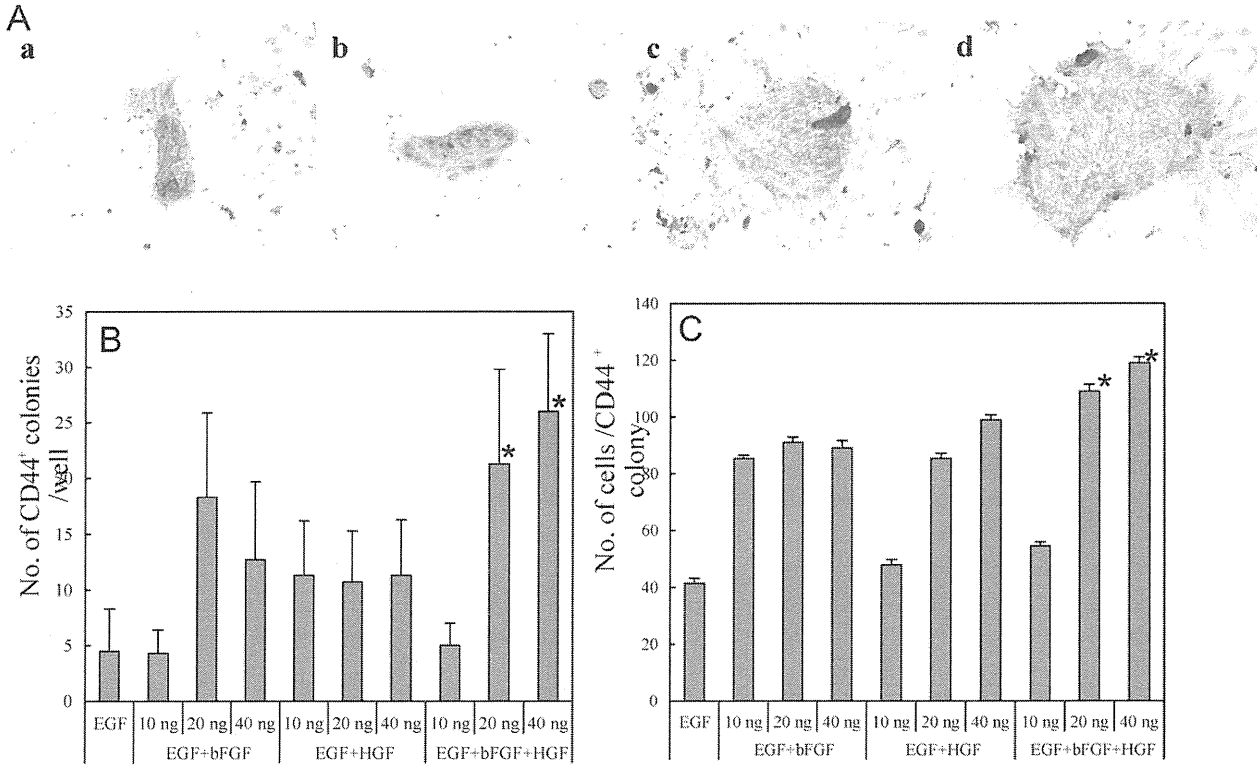


Fig. 2. Induction of CD44-positive cell colonies from sorted D2-Thy1$^+$ cells by treatment with EGF, bFGF, and/or HGF. Thy1$^+$ cells (1×10^5 viable cells/well) sorted from the GalN-D2 liver were plated on 12-well plates and cultured in medium supplemented with EGF (a), EGF+bFGF (b), EGF+HGF (c), and EGF+bFGF+HGF (d) for 10 days. Dose dependency of colony formation was examined. To identify colonies, ICC for CD44 was carried out. The number of CD44$^+$ cell colonies per well (B) and that of cells per colony (C) were measured. Asterisks shown in (B) and (C) indicate significance: $P < 0.05$, compared to EGF and 10 ng of EGF+bFGF+HGF.

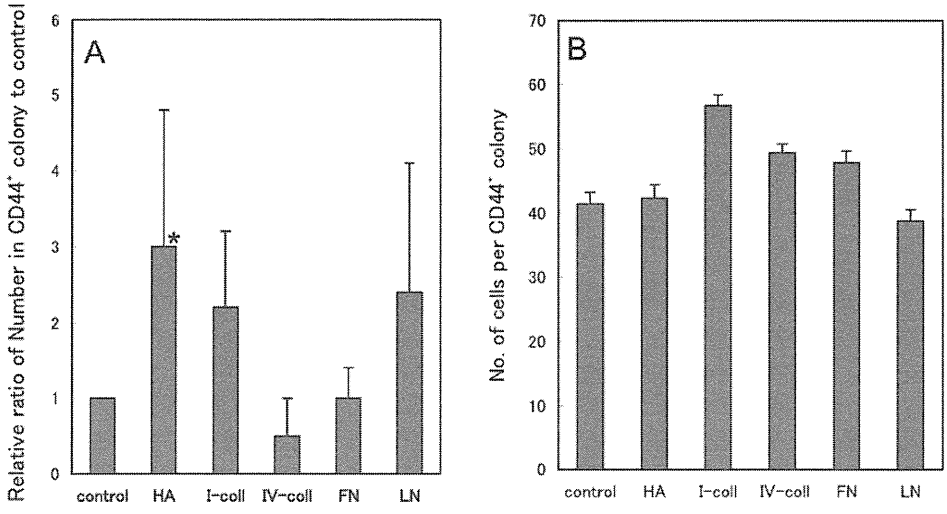


Fig. 3. Effects of ECM on colony formation of Thy1$^+$ cells were investigated. Thy1$^+$ cells were isolated from GalN-D2 rat livers and plated on dishes coated with hyaluronic acid (HA), type I collagen (I-coll), type IV collagen (IV-coll), fibronectin (FN), and laminin (LN). Noncoated dishes were used as controls. Cells were cultured in medium with EGF. To identify the colony, ICC for CD44 was carried out. The number of CD44$^+$ cell colonies per dish (A) and that of cells per colony (B) were measured. Asterisk shows significance: $P < 0.05$, control versus HA.

Induction of Epithelial Cell Colonies by Extracellular Matrix. Because CD44 is one of the receptors of hyaluronic acid (HA)[22,23] and because SHs can selectively proliferate on HA,[18] we investigated whether extracellular matrix (ECM) affected the frequency of emergence and phenotype of colonies derived from D2-Thy1$^+$ cells. Sorted D2-Thy1$^+$ cells were cultured on dishes coated with type I collagen, fibronectin, laminin, and HA, and ICC for CD44 was performed 10 days after plating. When cells were cultured in the medium supplemented with EGF, frequency of colony formation was significantly higher for cells on HA-coated dishes than for the control (Fig. 3A), but no difference was observed in the number of cells per colony among the dishes with each ECM (Fig. 3B).

Growth Ability and CD44 Expression of Cells Sorted From GalN-D3. Thy1$^+$CD44$^-$ (Thy1), Thy1$^+$CD44$^+$, and Thy1$^-$CD44$^+$ (CD44) cells sorted from a GalN-D3 liver were cultured in the medium with EGF for 10 days. Double ICC for CD44 and BrdU was carried out (Fig. 4A–C). The frequency of colony formation was more than four times higher for CD44 cells than for both Thy1 and Thy1$^+$CD44$^+$ cells (Fig. 4D), and the average number of cells per colony was significantly larger for CD44 cells than for Thy1 and Thy1$^+$CD44$^+$ cells (Fig. 4E). The percentages of BrdU$^+$ cells were approximately 70% and 80% in colonies derived from Thy1 and CD44 cells, respectively (Fig. 4A–C, F). Growth ability of Thy1$^+$CD44$^+$ cells was also intermediate between those of Thy1 and CD44 cells.

Intensity and the localization of CD44 varied among cells forming colonies. In spite of the origin of sorted cells, CD44 protein was usually expressed in cell membranes between cells (Fig. 4C). Some colonies consisted of cells with CD44 protein localized in both the cell membrane and cytoplasm (Fig. 4A, B). The latter type of colony was often observed in the culture of Thy1 cells. CD44 positivity of Thy1$^+$ cells in a colony was approximately 65% at day 5 and increased to approximately 80% at day 10 (Fig. 4G).

Next, to examine whether acquisition of CD44 expression in Thy1$^+$ cells was also correlated to growth ability of cells *in vivo*, cell transplantation was carried out. D2-Thy1$^+$, D3-Thy1$^+$CD44$^-$, D3-Thy1$^+$CD44$^+$, D3-Thy1$^-$CD44$^+$, and D4-CD44$^+$ cells (5×10^5 cells/rat) isolated from GalN-treated livers were intrasplenically transplanted into RET/PH-treated rats. One month after transplantation, the number of cells in foci derived from D4-CD44 was much larger than in those from Thy1-expressing cells (Fig. 4H, I). The growth rate of engrafted cells increased in correlation with the expression of CD44 and time after GalN treatment. In addition, no types of donor-derived (Y chromosome$^+$) cells, other than hepatocytes, could be found in recipient livers (Supporting Fig. 1).

Gene Expression of Hepatic Markers of Epithelial Cell Colonies Derived From D3-Thy1 Cells. To elucidate the characteristics of cells in colonies derived from D3-Thy1 cells, quantitative polymerase chain reaction (qPCR) of cells was performed for each colony, which was separated from the culture dish using a

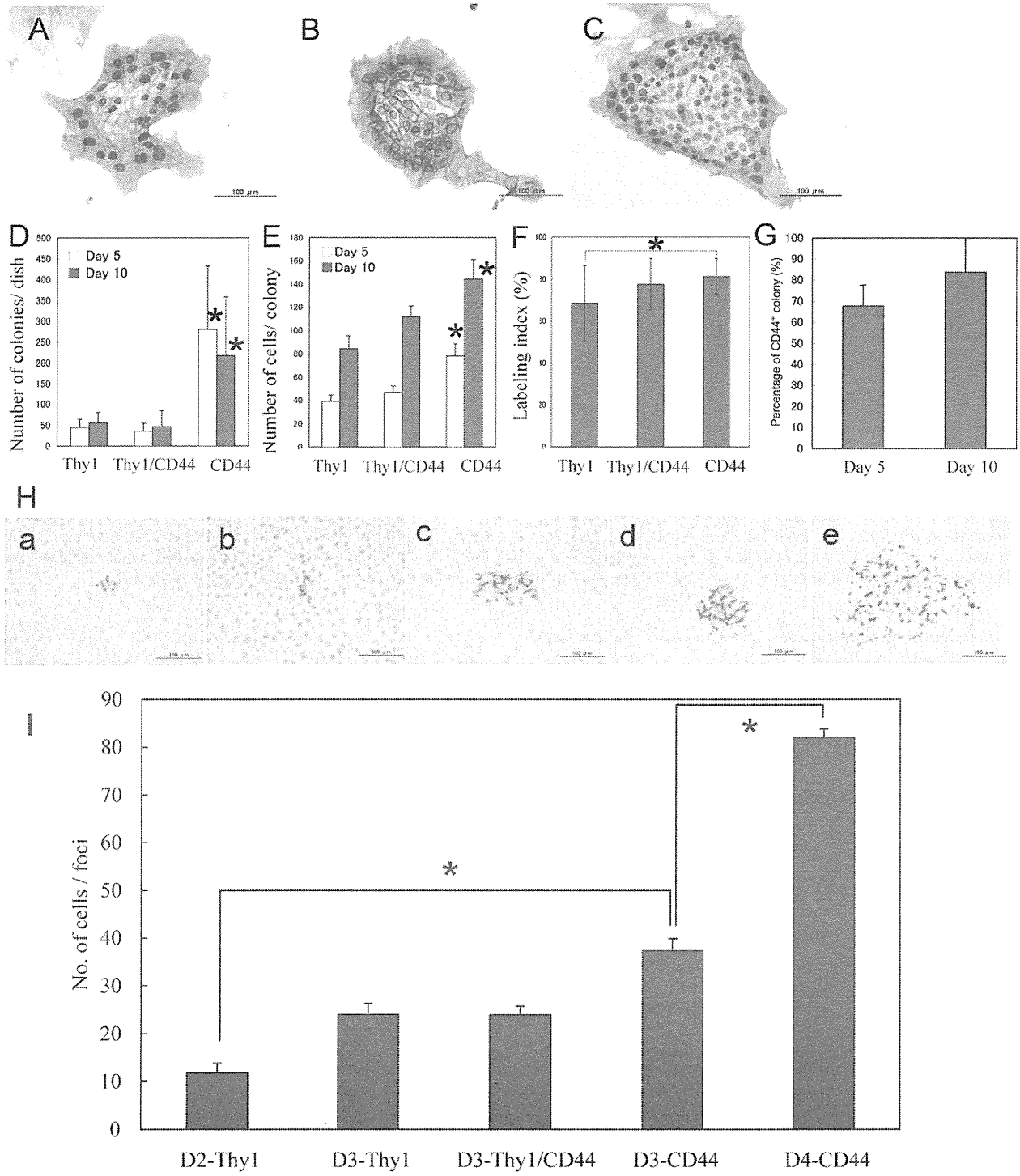


Fig. 4. Growth and CD44 positivity of epithelial cell colonies were examined in cultured cells. $Thy1^+$ (A), $Thy1^+/CD44^+$ (B), and $CD44^+$ cells (C) were isolated from GalN-D3 rat livers. Then, viable cells per dish (1×10^5) were plated on noncoated 35-mm culture dishes and cultured in the medium with EGF for 10 days. BrdU (40 μmol/L) was added to the medium 24 hours before fixation. At 5 and 10 days after plating, cells were fixed and double ICC for CD44 and BrdU was performed. CD44 and BrdU were stained violet and brown, respectively. Nuclei were counterstained with hematoxylin. The number of $CD44^+$ colony per dish (D), cells per colony (E), $BrdU^+$ cells per colony (F), and CD44 positivity of the colony derived from D2-$Thy1^+$ cells (G) were measured at 5 (white bar) and 10 days (gray bar) after plating. $CD44^+$ cell colonies were defined as colonies in which more than 90% of cells were stained with CD44. Asterisks show significant differences: $P < 0.05$. (D) $Thy1^+CD44^-$ and $Thy1^+CD44^+$ versus $Thy1^-CD44^+$ at days 5 and 10, (E) $Thy1^+CD44^-$ and $Thy1^+CD44^+$ versus $Thy1^-CD44^+$ at day 5, and $Thy1^+CD44^-$ versus $Thy1^-CD44^+$ at day 10, (F) $Thy1^+CD44^-$ versus $Thy1^-CD44^+$. Transplantation of isolated cells from GalN-treated livers into RET/PH-treated livers (H). Sorted GalN-D2-$Thy1^+$ (5×10^5 cells) (a), D3-$Thy1^+/CD44^-$ (b), D3-$Thy1^+/CD44^+$ (c), D3-$Thy1^-/CD44^+$ (d), or D4-$CD44^+$ cells (e) were intrasplenically transplanted into Ret/PH-treated rats. Thirty days after transplantation, livers were perfused with PBS, then sliced to make frozen sections. Tissues were enzymatically stained with DPPIV. DPPIV-positive cells are stained red. Nuclei are counterstained with hematoxylin (blue). The number of cells per focus was measured (I). Asterisks indicate significant differences: $P < 0.05$.

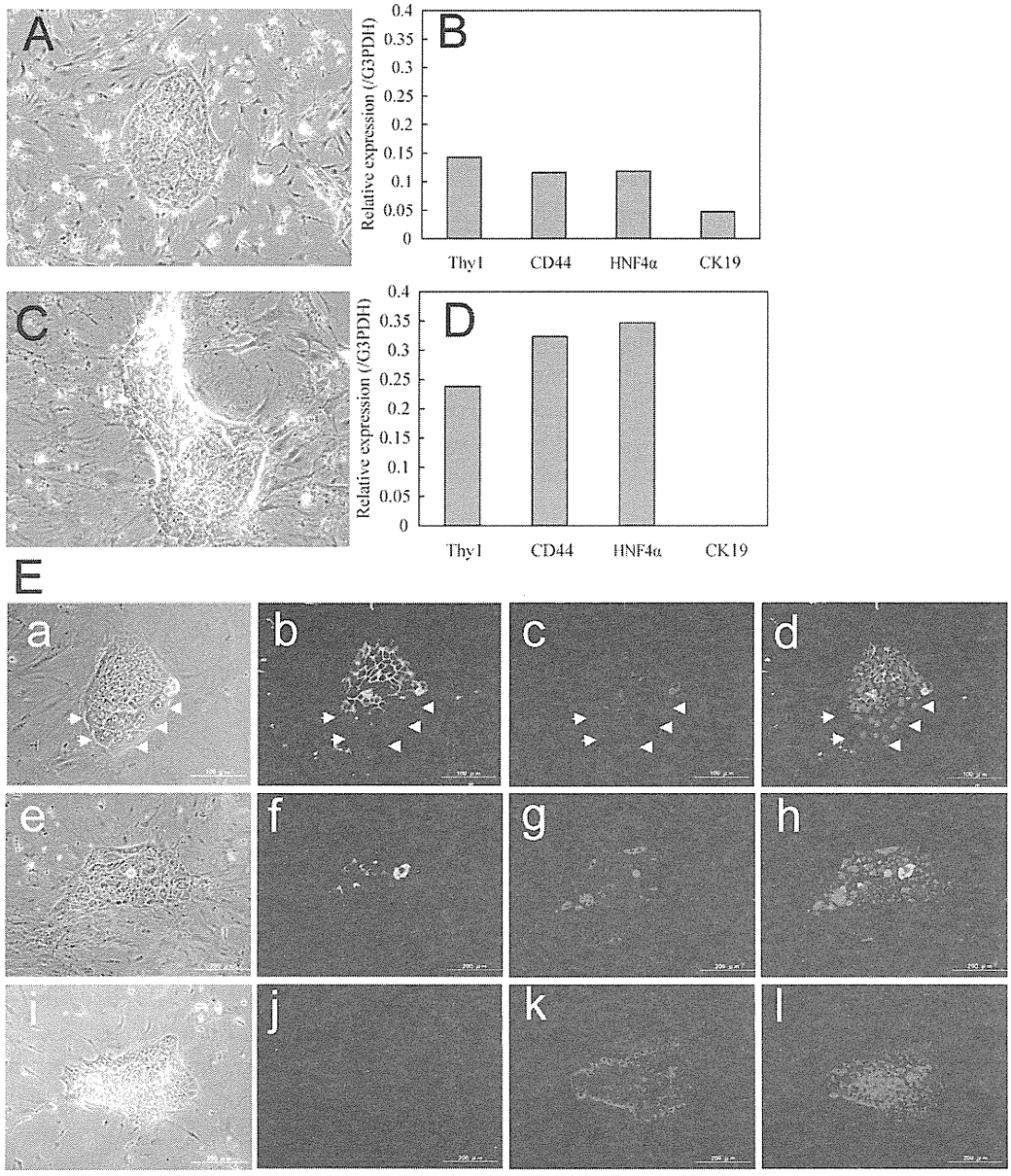


Fig. 5. Gene expression of the cells in each colony was measured by qPCR. Thy1^{+} cells sorted from GalN-D3 livers were cultured in the medium with EGF for 10 days. Each colony was separated from the dishes by using a cloning ring. Phase-contrast photos of all colonies were taken, then RNA of cells in the colonies was separated. qPCR was performed for each colony. Morphology (A and C) and gene expression (B and D) of representative colonies of CD44^{low} (A and B) and CD44^{high} (C and D) are shown. Cells were fixed at day 10, and double ICC for CD44/Thy1 (E, a-d) and CK-19/Alb (E, e-l) was performed. Although many cells in Thy1-derived colonies coexpressed CD44 and Thy1, some large cells (arrowheads) exhibited no expression of either protein (E, a-d). There were some colonies consisting of a mixture of Alb^{+} and CK-19^{+} cells (E, e-h), but most cells expressed Alb and only a few cells expressed CK-19 (E, i-l). Data for other colonies are shown in Supporting Fig. 2.

cloning ring. Phase-contrast photos were taken of every colony, and qPCR was performed. Gene-expression patterns of the colonies were roughly divided into two groups by the level of CD44 expression. Results for a representative colony in each group are shown in Fig. 5 (results for other colonies are shown in Supporting Fig. 2). Although intensity of gene expression varied among colonies, all colonies expressed CD44 messenger RNA (mRNA). Compared to cells in CD44^{low} colonies, those in CD44^{high} colonies showed not only relatively high expression of albumin and hepatocyte nuclear factor (HNF)-4α, but also suppression of CK-19 expression. Although coexpression of CD44 and Thy1 was observed in many Thy1-derived colonies, some large cells (arrowheads) exhibited no expression of either protein (Fig. 5E, a–d). There were colonies consisting of a mixture of albumin (Alb)$^{+}$ and CK-19^{+} cells (Fig. 5E, e–h), though most cells expressed

Alb and only a few cells exhibited CK-19 (Fig. 5E, i–l). To induce maturation of cells in Thy1-derived colonies, cells were treated with Matrigel. The treatment dramatically decreased Thy1 expression and increased levels of both HNF-4α and Alb (Supporting Fig. 3A). In addition, a marked increase of Alb secretion was also observed in cells with Matrigel (Supporting Fig. 3B). However, neither CD44 nor CK-19 expression was changed by the treatment.

Induction of Maturation in Cultured Cells. Next, we examined whether the newly generated hepatocytes could reconstruct hepatic organoids with highly differentiated functions. To enhance the organoid formation of colonies, colonies derived from D3-Thy1, CD44, and SHs from a healthy liver were replated on collagen-coated dishes to increase their density. In contrast to the Matrigel treatment, this procedure resulted in natural organoid formation, which consisted of piled-up cells with BCs. The expression of CCAAT/enhancer-binding protein (C/EBP)-α was ICC examined, and expression of genes related to hepatic differentiated functions was investigated by qPCR. In addition, to certify the function of the newly formed BCs, FD was added to the culture medium and the ability to secrete fluorescence into BCs was examined.

In spite of their origins, some cells in colonies became large and piled up with time after replating. Morphologically, BCs and cyst-like structures were observed in colonies, similar to those in colonies formed by SHs derived from healthy rat liver.[13,19,20] However, ICC for C/EBP-α revealed that the numbers of positive nuclei in Thy1- and CD44-derived colonies were smaller than in SH-derived colonies (Figs. 6A, B). Results of qPCR for each colony derived from Thy1, CD44, and SHs revealed that gene expression of cytochrome P450 1A2 (CYP1A2), tryptophan 2,3-dioxygenase (TDO), and carbamoylphosphate synthetase I (CPS-I), which are regarded as indicators for differentiated hepatic functions, was significantly higher in SH than CD44 or Thy1 (Fig. 6C). However, expression of tyrosine aminotransferase (TAT) was not different among cells.

In a SH-derived colony, fluorescence was secreted into BCs and accumulated in cysts (Fig. 7, e,f). The networks of BCs were well developed, corresponding to the regions of piled-up cells. On the other hand, in the colonies derived from both Thy1 (Fig. 7A, a,b) and CD44 cells (Figs. 7A, c,d), part of the region consisting of piled-up cells had a green, patch-like appearance. This phenomenon indicated the retention of fluorescence in the cytoplasm. In addition, to quantitatively compare structural differentiation among cells, the total length of BCs was measured in each colony. Total length of BCs was significantly larger in the SH-derived colony than in the Thy1- and CD44-derived colonies (Fig. 7B). These results suggested that newly generated hepatocytes derived from Thy1 and CD44 were not as mature as those from SHs.

Discussion

Hepatocytic Differentiation of Thy1-Positive Cells. Thy1 was first identified as a marker of oval cells by Petersen et al.[24] and then widely used in experiments with HPCs. Recently, a question was raised about the validity of Thy1 as a marker for oval cells.[25,26] It was reported that Thy1 was not a marker of oval cells, but of hepatic myofibroblasts and/or stellate cells. Although the issue regarding whether Thy1 is a marker for hepatic stem/progenitors is open to debate, we recently found that some $Thy1^+$ cells isolated from GalN-treated livers differentiated into $CD44^+$ hepatocytes through $Thy1^+CD44^+$ cells.[15] These results suggested that the population of $Thy1^+$ cells was heterogeneous, and that it contained putative hepatic stem cells possessing the ability to differentiate into hepatocytes. Interestingly, colony formation was clearly observed in the culture of cells from GalN-D3, whereas it was rarely observed in $Thy1^+$ cells isolated from GalN-D2, suggesting that $Thy1^+$ cells became the hepatic lineage between D2 and D3. In the present experiment, we demonstrated that EGF, fibroblast growth factor (FGF), and HGF might trigger the commitment to the hepatic lineage of $Thy1^+$ cells, some of which possess capability as putative stem cells. It has been reported that transforming growth factor (TGF)-α, HGF, and FGF play important roles in stem/progenitor cell-mediated liver regeneration. Indeed, the growth factors are transcriptionally upregulated during the period of active proliferation and differentiation of progenitor cells in rat liver[27–29] and appear to drive the early proliferation of the progenitor cell compartment.[27,30] Interestingly, it has been reported that stellate cells, which proliferate concomitantly and in close contact with progenitor cells,[31] appear to be the main source of TGF-α, HGF, and acidic FGF, whereas the corresponding cognate receptors are strongly expressed in progenitor cells, suggesting that the regulation of progenitor cell proliferation and differentiation by growth factors occurs primarily in a paracrine manner.[2,5] On the other hand, it is well known that priming factors are necessary for the emergence and proliferation of HPCs.[5,7] A correlation between the severity of liver disease and the magnitude

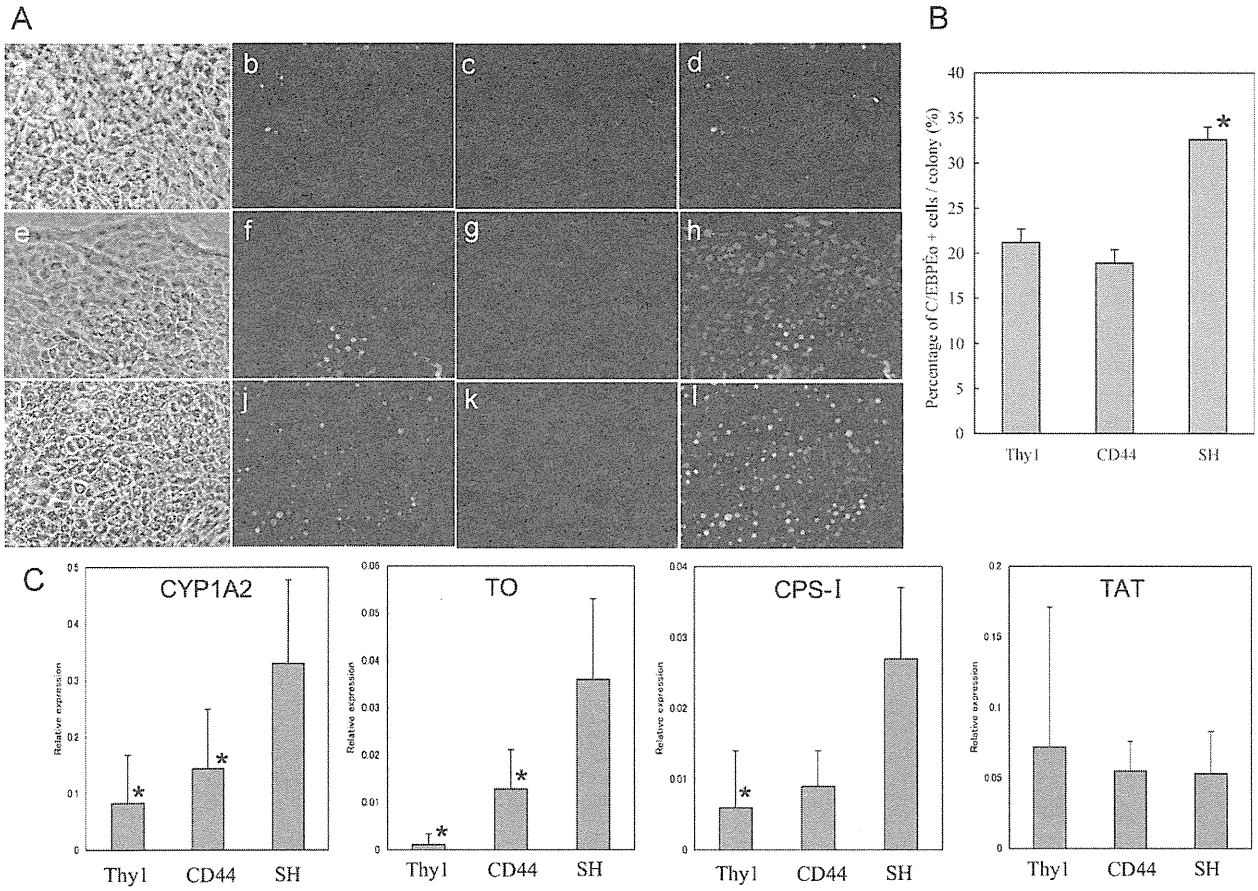


Fig. 6. To enhance organoid formation of the colonies, colonies derived from GalN-D3 Thy1 cells (A, a-d), CD44 cells (A, e-h), and a healthy liver (A, i-l) were replated on new collagen-coated dishes at 10 days after plating to increase the density of the colonies. Cells were cultured for more than 14 days after replating. This procedure resulted in natural organoid formation, which consisted of piled-up (matured) cells. The expression of C/EBP-α (A,b,f,j) and CD44 (A,c,g,k) was ICC observed and the percentage of C/EBP-α^+ cells per colony was measured (B). The gene expression of CYP1A2, TDO, CPS-I, and TAT in Thy1, CD44 cells, and SHs derived from a healthy liver was analyzed by real-time PCR (C). The scale is shown as a value relative to that of MH. Asterisks indicate a significant difference: $P < 0.05$, SH versus Thy1 or CD44.

of the response of hepatic progenitor cells has been reported and inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and interferon-gamma (INF-γ), were suggested to play central roles as priming factors in rodents.[32–36] However, in the present experiment, TNF-α, IL-6, and INF-γ could not induce the epithelial differentiation of Thy1$^+$ cells or enhance their expansion. This might be because D2-Thy1$^+$ cells have already been primed by the inflammation induced by GalN.

Expression of CD44 in Thy1-Derived Cells. In this experiment, we demonstrated that the growth and degree of hepatocytic differentiation of Thy1$^+$ cells were correlated with the expression of CD44. The results of GeneChip analysis demonstrated that the expression of genes related to hepatocytic differentiation, which were absent in GalN-D2-Thy1 cells, progressively increased in the order D3-Thy1, D3-Thy1$^+$CD44$^+$, and D4-CD44 cells. Although results of qPCR showed that the degree of expression of the genes of interest was different among epithelial colonies derived from D3-Thy1 cells, the cells in the CD44^{high} colonies showed high expression of Alb and HNF-4α, compared to cells in CD44^{low} colonies. In fact, CD44 expression in Thy1$^+$ cells increased with time in culture.

Acquisition of CD44 expression in Thy1$^+$ cells was also correlated with growth ability of cells. Growth of cells in CD44$^+$ cell-derived colonies was clearly faster than that of cells in colonies derived from Thy1-expressing cells. High growth activity of CD44$^+$ cells was also shown in the cell transplantation experiment. One month after transplantation, the number of cells in the foci derived from D4-CD44 was much larger than in those from Thy1-expressing cells. In general, the growth speed of cells shows an inverse correlation with degree of cell differentiation, and less-differentiated cells can proliferate much faster than differentiated cells. However, in the present experiment, although

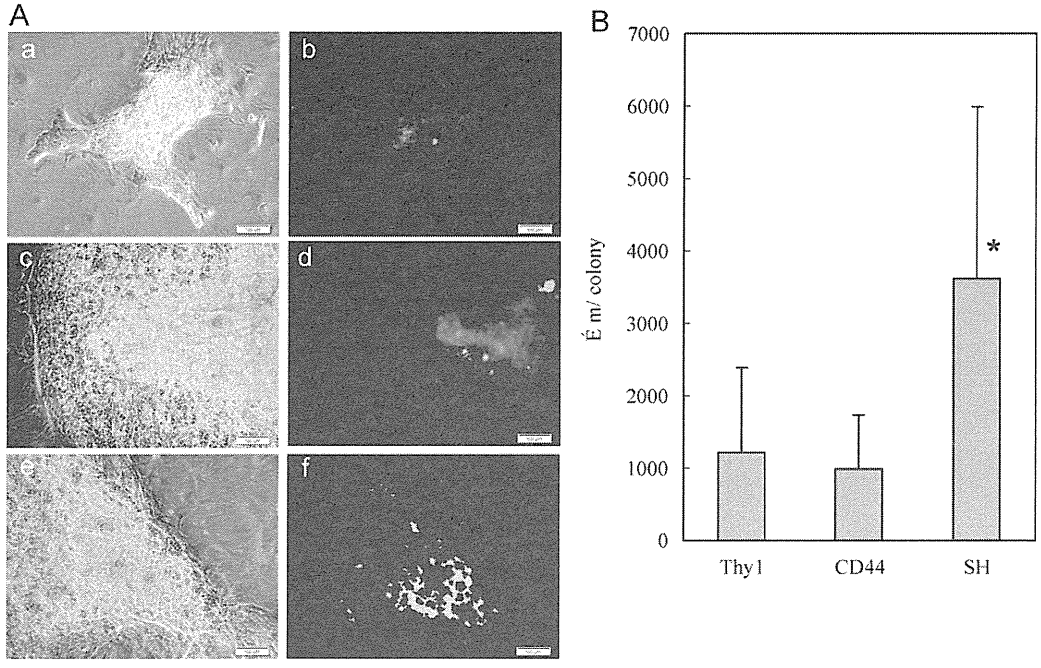


Fig. 7. To demonstrate the function of the newly formed BCs in hepatic organoids, FD was added to the culture medium and the ability to secrete fluorescence into BCs was examined. A part of the region consisting of piled-up cells in the colonies derived from both Thy1 (A, a,b) and CD44 cells (A, c,d) shows a green, patch-like appearance. Fluorescence was secreted into BCs and accumulated in the cysts in an SH-derived colony. To compare the development of BCs, the total length of BCs was measured in each colony (B). Total length of BCs was significantly larger in the SH-derived colony than in the Thy1- and CD44-derived colonies. Asterisk indicates a significant difference: $P < 0.05$, SH versus Thy1 and CD44.

$CD44^+$ cells were more differentiated than $Thy1^+$ cells, growth speed of $CD44^+$ cells was higher than that of $CD44^-$ cells (data not shown). At present, we cannot explain these findings, and further experiments will be required to clarify the regulatory mechanism of CD44 expression.

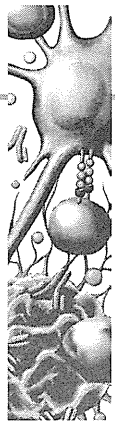
Restricted Maturation of HPCs. We previously reported that SHs derived from the healthy liver could spontaneously differentiate into hepatocytes that showed typical features of MHs and reconstructed three-dimensional (3D) structures by interacting with hepatic nonparenchymal cells.[11] In 3D structures, a complicated network of BCs is formed, and, when FD is added, fluorescence is secreted to BCs and expands all over the colony.[11,20] In the present experiment, despite their origins, cells could become large and pile up to form 3D structures that were morphologically similar to the hepatic organoids previously reported.[11,13] However, fluorescence was mostly retained in the cytoplasm of cells derived from both $Thy1^+$ and $CD44^+$ cells. Cytoplasmic retention of fluorescence indicates that cellular polarity is not well established, so that BCs cannot be well reconstructed. The short length of BCs also showed the incomplete maturation of both Thy1- and CD44-derived cells. Furthermore, compared to the organoids derived from SHs, CD44 expression remained and the ratio of C/EBP-α^+ cells was lower in organoids derived from both $Thy1^+$ and $CD44^+$ cells. The lower expression of CYP1A2, TDO, and CPS-I genes in organoids from Thy1 and CD44 than in those from SHs also indicated the immaturity of stem/progenitor cell-derived hepatocytes. These results demonstrated that the newly generated hepatocytes derived from $Thy1^+$ and $CD44^+$ cells might not have acquired the same highly differentiated functions as MHs. On the other hand, we previously reported that, compared to MHs, the repopulation efficiency of the liver by transplanted $Thy1^+$ cells was very low, and that most Thy1-derived foci disappeared within 2 months after transplantation.[16] Similar results were shown for the transplantation of $CD44^+$ cells. Those results indicate that HPCs may not be able to survive for a long time. Detailed histological analysis at 2 weeks after transplantation revealed that the percentage of C/EBP-α^+ cells in the early CD44-derived foci was lower than that in MH-derived foci. In addition, the size and shape of the cells and the distribution of the $DPPIV^+$ membrane were more irregular in Thy1- and CD44-derived foci than in MH-derived foci, which meant that BCs were not connected among cells, and that sinusoids were indistinct.[16] These cell-transplantation results may reflect the *in vitro* data shown in the present experiments. Shafritz et al.[6] summarized previous transplantation experiments using HPCs, and found that cells showed very low efficiency

of engraftment and repopulation, regardless of the condition of the recipient liver. Thus, hepatocytes induced from embryonic stem cells and other stem/progenitor cells have not yet matured to the stage at which they can efficiently repopulate the liver of an adult. In other words, to use HPCs in regenerative medicine, the state of differentiation of the cells used for cell transplantation may be very important, and a procedure for assessment of the maturation should be immediately developed.

Acknowledgments: The authors thank Ms. Minako Kuwano and Ms. Yumiko Tsukamoto for their technical assistance. The authors also thank Mr. Kim Barrymore for his help with the manuscript for this article.

References

1. Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. HEPATOLOGY 2001;33:738-750.
2. Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. HEPATOLOGY 2004;39:1477-1487.
3. Michalopoulos GK. Liver regeneration: alternative epithelial pathways. Int J Biochem Cell Biol 2011;43:173-179.
4. Farber E. Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorene, and 3'-methy-4-dimethylaminoazobenzene. Cancer Res 1956;16:142-149.
5. Santoni-Rugiu E, Jelnes P, Thorgeirsson SS, Bisgaard HC. Progenitor cells in liver regeneration: molecular responses controlling their activation and expansion. APMIS 2005;113:876-902.
6. Shafritz DA, Oertel M, Menthena A, Nierhoff D, Dabeva MD. Liver stem cells and prospects for liver reconstitution by transplanted cells. HEPATOLOGY 2006;43:S89-S98.
7. Bird TG, Lorenzini S, Forbes SJ. Activation of stem cells in hepatic diseases. Cell Tissue Res 2008;331:283-300.
8. Lemire JM, Shiojiri N, Fausto N. Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol 1991;139:535-552.
9. Evarts RP, Hu Z, Omori N, Omori M, Marsden ER, Thorgeirsson SS. Precursor-product relationship between oval cells and hepatocytes: comparison between tritiated thymidine and bromodeoxyuridine as tracers. Carcinogenesis 1996;17:2143-2151.
10. Mitaka T, Kojima T, Mizuguchi T, Mochizuki Y. Growth and maturation of small hepatocytes isolated from adult liver. Biochem Biophys Res Commun 1995;214:310-317.
11. Mitaka T, Sato F, Mizuguchi T, Yokono T, Mochizuki Y. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. HEPATOLOGY 1999;29:111-125.
12. Sasaki K, Kon J, Mizuguchi T, Chen Q, Ooe H, Oshima H, et al. Proliferation of hepatocyte progenitor cells isolated from adult human livers in serum-free medium. Cell Transplant 2008;17:1221-1230.
13. Sugimoto S, Mitaka T, Ikeda S, Harada K, Ikai I, Yamaoka Y, et al. Morphological changes induced by extracellular matrix are correlated with maturation of rat small hepatocytes. J Cell Biochem 2002;87:16-28.
14. Kon J, Ooe H, Oshima H, Kikkawa Y, Mitaka T. Expression of CD44 in rat hepatic progenitor cells. J Hepatol 2006;45:90-98.
15. Kon J, Ichinohe N, Ooe H Chen Q, Sasaki K, Mitaka T. Thy1-positive cells have bipotential ability to differentiate into hepatocytes and biliary epithelial cells in galactosamine-induced rat liver regeneration. Am J Pathol 2009;175:2362-2371.
16. Ichinohe N, Kon J, Sasaki K, Nakamura Y, Ooe H, Tanimizu N, et al. Growth ability and repopulation efficiency of transplanted hepatic stem, progenitor cells, and mature hepatocytes in retrorsine-treated rat livers. Cell Transplant 2012;21:11-22.
17. Gordon GJ, Coleman WB, Grisham JW. Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats. Am J Pathol 2000;157:771-786.
18. Chen Q, Kon J, Ooe H, Sasaki K, Mitaka T. Selective proliferation of rat hepatocyte progenitor cells in serum-free culture. Nat Protoc 2007;2:1197-1205.
19. Oshima H, Kon J, Ooe H, Hirata K, Mitaka T. Functional expression of organic anion transporters in hepatic organoids reconstructed by rat small hepatocytes. J Cell Biochem 2008;104:68-81.
20. Sudo R, Ikeda S, Sugimoto S, Harada K, Hirata K, Tanishita K, et al. Bile canalicular formation in hepatic organoid reconstructed by rat small hepatocytes and nonparenchymal cells. J Cell Physiol 2004;199:252-261.
21. Tanimizu N, Nishikawa M, Saito H, Tsujimura T, Miyajima A. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J Cell Sci 2003;116:1775-1786.
22. Goodison S, Urquidi V, Tarin D. CD44 cell adhesion molecules. Mol Pathol 1999;52:189-196.
23. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003;4:33-45.
24. Petersen BE, Goff JP, Greenberger JS, Michalopoulos GK. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. HEPATOLOGY 1998;27:433-445.
25. Dezső K, Jelnes P, László V, Baghy K, Bödör C, Paku S, et al. Thy-1 is expressed in hepatic myofibroblasts and not oval cells in stem cell-mediated liver regeneration. Am J Pathol 2007;171:1529-1537.
26. Dudas J, Mansuroglu T, Batusic D, Saile B, Ramadori G. Thy-1 is an *in vivo* and *in vitro* marker of liver myofibroblasts. Cell Tissue Res 2007;329:503-514.
27. Evarts RP, Hu Z, Fujio K, Marsden ER, Thorgeirsson SS. Activation of hepatic stem cell compartment in the rat: role of transforming growth factor α, hepatocyte growth factor, and acidic fibroblast growth factor in early proliferation. Cell Growth Differ 1993;4:555-561.
28. Marsden ER, Hu Z, Fujio K, Nakatsukasa H, Thorgeirsson SS, Evarts RP. Expression of acidic fibroblast growth factor in regenerating liver and during hepatic differentiation. Lab Invest 1992;67:427-433.
29. Hu Z, Evarts RP, Fujio K, Omori N, Omori M, Marsden ER, et al. Expression of transforming growth factor alpha/epidermal growth factor receptor, hepatocyte growth factor/c-*met*, and acidic fibroblast growth factor/fibroblast growth factor receptors during hepatocarcinogenesis. Carcinogenesis 1996;17:931-938.
30. Nagy P, Bisgaard HC, Santoni-Rugiu E, Thorgeirsson SS. *In vivo* infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene. HEPATOLOGY 1996;23:71-79.
31. Paku S, Schnur J, Nagy P, Thorgeirsson SS. Origin and structural evolution of the early proliferating oval cells in rat liver. Am J Pathol 2001;158:1313-1323.
32. Nagy P, Kiss A, Schnur J, Thorgeirsson SS. Dexamethasone inhibits the proliferation of hepatocytes and oval cells but not bile duct cells in rat liver. HEPATOLOGY 1998;28:423-429.
33. Bisgaard HC, Müller S, Nagy P, Rasmussen LJ, Thorgeirsson SS. Modulation of the gene network connected to interferon-γ in liver regeneration from oval cells. Am J Pathol 1999;155:1075-1085.
34. Knight B, Yeoh GC, Husk KL, Ly T, Abraham LJ, Yu C, et al. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. J Exp Med 2000;192:1809-1818.
35. Knight B, Matthews VB, Akhurst B, Croager EJ, Klinken E, Abraham LJ, et al. Liver inflammation and cytokine production, but not acute phase protein synthesis, accompany the adult liver progenitor (oval) cell response to chronic liver injury. Immunol Cell Biol 2005;83:364-374.
36. Matthews VB, Klinken E, Yeoh GC. Direct effects of interleukin-6 on liver progenitor oval cells in culture. Wound Repair Regen 2004;12:650-656.



Heat-shock proteins as endogenous ligands building a bridge between innate and adaptive immunity

There has been growing evidence that heat-shock protein (HSP) functions as an endogenous immunomodulator for innate and adaptive immune responses. Since HSPs inherently act as chaperones within cells, passive release (e.g., by cell necrosis) and active release (including release by secretion in the form of an exosome) have been suggested as mechanisms of HSP release into the extracellular milieu. Such extracellular HSPs have been shown to be activators of innate immune responses through Toll-like receptors. However, it has also been suggested that HSPs augment the ability of associated innate ligands such as lipopolysaccharides to stimulate cytokine production and dendritic cell maturation. More interestingly, a recent study has demonstrated that innate immune responses elicited by danger signals were regulated spatiotemporally and that can be manipulated by HSPs, thereby controlling immune responses. We will discuss how spatiotemporal regulation of HSP-chaperoned molecules within antigen-presenting cells affects adaptive immunity via antigen cross-presentation and innate immune responses. Precise analysis of HSP biology should lead to the establishment of effective HSP-based immunotherapy.

KEYWORDS: cross-presentation danger signal dendritic cell heat-shock protein Toll-like receptor

Yasuaki Tamura*[1], Toshihiko Torigoe[1], Kazuharu Kukita[1,2], Keita Saito[2], Koichi Okuya[2], Goro Kutomi[2], Koichi Hirata[2] & Noriyuki Sato[1]

[1]Department of Pathology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan
[2]Department of Surgery, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan
*Author for correspondence: ytamura@sapmed.ac.jp

Heat-shock proteins (HSPs), highly conserved across species, are generally considered to be intracellular proteins that have protective functions in situations of cellular stress [1]. A wide variety of stressful stimuli such as heat shock, UV radiation, and viral or bacterial infections induce a substantial increase in intracellular HSP synthesis. The main functions of HSPs are to act as chaperones of nascent or aberrantly folded proteins [1,2]. HSPs have also been shown to have important functions in the mammalian immune system. Srivastava's group first identified the endoplasmic reticulum (ER)-resident HSP gp96 as a tumor antigen [3,4]. Immunization of mice with gp96 isolated from tumors induced an antitumor immune response through the induction of tumor-specific cytotoxic T lymphocytes (CTLs). This immunogenicity is based on antigenic peptides that are associated with gp96 molecules, and peptide-deprived HSP complexes lose their specific immunogenicity. Following these observations, Srivastava's group further demonstrated that immunization with tumor-derived Hsp70 and Hsp90 also elicited tumor-specific CTL responses [5,6]. In particular, they showed that Hsp70 bound tumor-specific antigenic peptides because Hsp70 treated with ATP released bound peptides, resulting in loss of their immunogenicity [5]. Noessner *et al.* also demonstrated that tumor-derived Hsp70 bound a tumor antigen (tyrosinase) peptide [7]. Moreover, the role of extracelluar HSPs in the stimulation of innate immunity has drawn much attention in recent years. We will discuss the current view of this unique feature of HSPs in the regulation of innate and adaptive immune responses.

HSPs as key players of the immune system

Recently obtained evidence indicates that extracellular HSPs play an important role in the induction of innate immune responses [2]. Since HSPs do not have a canonical signal sequence, it has been suggested that HSPs may be released via an active secretion mechanism or from cells undergoing necrosis [2,8]. The resultant extracellular HSPs may then interact with dendritic cell (DCs) or macrophages, resulting in the activation of innate immune responses including maturation of DCs and secretion of proinflammatory cytokines and chemokines through Toll-like receptor (TLR) activation [9,10]. This unique feature of certain HSPs is termed 'chaperokine', which describes that extracellular HSPs act as both chaperones and cytokines [11]. By contrast, regarding the adaptive immunity, HSPs have another unique feature acting as a cross-presentation inducer. DCs have a unique ability to take up, process and present exogenous antigens in association with MHC class I molecules, termed cross-presentation [12]. The cross-presentation

Future Medicine part of fsg

plays a pivotal role in priming antigen-specific naive T-cell responses to tumor cells and virus-infected cells that cannot access the classical pathway for MHC class I presentation. It has been shown that HSPs can be released in the form of complexes with antigenic peptides and that these HSPs–peptide complexes are taken up by antigen-presenting cells (APCs) such as DCs through specific receptors expressed on APCs, leading to cross-presentation [4,8,13]. Thus, extracellular HSPs act as chaperokines for activation of innate immunity as well as enhancers for adaptive immunity of antigen-specific T-cell induction (summarized in Table 1).

These findings led to the idea that APCs bear HSP-specific receptors on the cell surface. Following the identification of CD91/low-density lipoprotein receptor-related protein-1 (LRP-1) as a gp96 receptor [14], many receptors including members of the TLR and scavenger receptor (SR) families have been shown to be HSP receptors.

Considering the establishment of HSP-based cancer immunotherapy, it is very important to develop the orchestrated link between innate and adaptive immunity via specific HSP receptors. Therefore, we next overview the HSP receptors expressed on APCs.

HSP receptors

HSPs interact with a range of receptors expressed on target cells. These receptors can be divided into two groups: TLRs and SRs [15]. TLRs are major pattern-recognition receptors (PRRs) and 11 have been identified in mammals. Two TLRs, TLR2 and TLR4, have been demonstrated to function as receptors for Hsp60, Hsp70 and gp96, leading to NF-κB activation [16–18]. In addition, the cell-surface protein CD14 required for lipopolysaccharide (LPS)-mediated TLR4 activation, has been shown to be required for Hsp70-mediated induction of cytokines TNF-α, IL-1β and IL-6 [10]. Lehner *et al.* showed that immunological consequences of Hsp72 can be localized to specific domains of the Hsp72 molecule. The C-terminal portion of Hsp72 (amino acids 359-610) stimulates production of chemokines, IL-12, TNF-α and NO, induces Th1 polalization and stimulates the maturarion of DCs. The N-terminal ATPase domain (amino acids 1-358) largely lacks these functions [19]. Wheeler *et al.* have also demonstrated that the C-terminal region of Hsp72 serves as an activator for macrophages to produce TNF-α. These effects did not seem to result from LPS contamination [20]. However, some studies have suggested that these interactions between Hsp70 and TLR are not likely to be exerted through the direct binding of Hsp70 to CD14, TLR2 or TLR4, as cells stably transfected with CD14, TLR2 or TLR4 do not bind avidly to Hsp70 [21]. These findings suggest that low-affinity interactions may be involved in TLR activation by Hsp70. A previous study showed that TLR activation by Hsp60 requires the internalization of the Hsp60; therefore, experiments using cells with simple TLR gene overexpression may thus be inadequate to assess direct HSP–TLR binding [18,22].

SRs constitute the other family of PRRs. These are receptors for chemically modified

Table 1. Classification and function of major mammalian heat-shock proteins in immunological fields.

Family	HSPs	Intracellular localization	Cross-presentation	Induction of inflammatory cytokine
Small HSP	Hsp25/27/28	Cytosol	ND	ND
Hsp40	Hsp40	Cytosol	ND	ND
Hsp47	Hsp47	ER	ND	ND
Hsp60	Hsp60	Mitochondria	ND	Enhance [19]
Hsp70	Hsp70	Cytosol	Enhance [5,6,7,13]	Enhance [9,11,16], inhibit [81]
	Hsc70	Cytosol	ND	ND
	mtHsp70	Mitochondria	ND	ND
	BiP (Grp78)	ER	Enhance [82]	ND
Hsp90	Hsp90	Cytosol	Enhance [74,75]	Enhance [17]
	Gp96/Grp94	ER	Enhance [14,23]	Enhance [17]
Hsp100	Hsp105/Hsp110	Cytosol	Enhance [83]	Enhance [84]
	ORP150/Grp170	ER	Enhance [80,85]	ND

ER: Endoplasmic reticulum; HSP: Heat-shock protein; ND: Not determined.

forms of lipoproteins, including oxidized and acetylated low-density lipoproteins. The SR family is divided into eight subclasses (A to H), and many receptors belonging to this family are expressed on the surface of APCs. The oxidized low-density lipoprotein-binding protein CD91/LRP has been shown to be a common receptor for Hsp60, Hsp70, Gp96 and calreticulin [23]. However, Theriault showed that the difference of Hsp70 binding to CD91-positive and -negative cells was minimal [21]. Therefore, Hsp70 binding to CD91 may be a low-affinity interaction or may be indirect. It has been demonstrated that Hsp70 can interact with at least three members of the SR family, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) [24], SR expressed by endothelial cells-1 (SREC-1) [25], and fasciclin, EGF-like, laminin-type EGF-like and link domain-containing SR-1 (FEEL-1) [25]. Hsp70 can be bound at high affinity by these SRs and internalized. Both Hsp90 and Hsp60 can also bind to LOX-1. In addition, gp96 and calreticulin show significant affinity to SR-A1 and SREC-1 and are internalized by these receptors [26,27]. However, HSP (gp96) binding to SR-A1 is immunosuppressive [28], whereas LOX-1 mediates Hsp70 immunogenicity and antigen presentation [24]. Therefore, detailed studies of each receptor–HSP interaction are essential to determine the effects of HSPs on immune responses.

Mechanism of HSP release from cells

As described above, immunization with HSP–peptide complexes elicits antitumor immune responses via cross-presentation by APCs. Do immune responses induced by extracellular HSP-mediated antigen cross-presentation actually take place *in vivo*? Because HSPs are inherently intracellular proteins, some mechanism for the release of endogenous HSPs into the extracellular space must exist. However, HSPs are not secreted via the classical pathway because their sequences encode no secretion leader signals. In fact, as described later, it has been shown that the export of HSPs to the extracellular space could not be blocked by typical inhibitors of the ER–Golgi pathway, such as brefeldin A. Currently, two mechanisms are considered to result in the release of HSPs from cells: passive release mechanisms such as necrotic cell death caused by exposure to hypoxia, severe trauma, surgery and lytic virus infection [29]; and active release mechanisms involving nonclassical protein release pathways [8,30–32].

Passive release mechanism

Release of intracellular proteins involves cell lysis and this may occur in pathological conditions that give rise to necrosis [29]. Basu and coworkers reported that HSPs, including gp96, Hsp90, Hsp72 and calreticulin, are released from necrotic cells but not from apoptotic cells [17]. Necrotic cell death results in the release of intracellular contents into the extracellular space, thereby liberating HSPs. Their findings make cell necrosis an attractive explanation for the mechanism by which HSPs are released into the extracellular milieu as an intrinsic immunological messenger termed endogenous danger signal as described later.

Active release mechanism

An active release mechanism has been suggested as an additional mechanism to the passive release hypothesis. Asea *et al.* showed that IFN-γ and IL-10 induce the active release of constitutively expressed Hsp70 (Hsc73) as well as Hsp72 from tumors under conditions that will not induce cell death [30]. Since IFN-γ and IL-10 are thought to exist at high concentrations within inflammatory foci, these cytokines may mediate the active release of Hsc73 and Hsp72 *in vivo* [31]. Moreover, Asea's group showed that whereas some extracellular Hsp72 could be found as free Hsp72, a proportion of extracellular Hsp72 was released within exosomes [31,32]. Exosomes are internal vesicles of multivesicular bodies released into the extracelluar milieu upon fusion of an multivesicular bodies with the cell surface. Additionally, they showed that Hsp72 was released by a nonclassical protein transport pathway and that intact surface membrane lipid rafts were required for efficient stress-induced Hsp72 release. Mambula *et al.* also demonstrated that a prostatic cancer cell line secreted Hsp72 via an endolysosomal pathway [8]. Furthermore, Fleshner *et al.* demonstrated another possible mechanism for Hsp72 release under the condition of psychological stress [33]. They proposed that activation of the sympathetic nervous system by stimuli such as stressor exposure results in the release of norepinephrine and subsequent activation of α1-adrenergic receptors. Stimulation of α1-adrenergic receptors results in an increase in intracellular Ca^{2+}, which may stimulate the release of exosomes containing Hsp72. More recently, Vega *et al.* [34] and De Maio [35] have demonstrated that Hsp72 is inserted into the plasma membrane of cells after stress, which may be formed by inverse evagination. It was also shown that

Hsp72 was released into the extracellular space in a membrane-associated form (i.e., exosome) that could act as a danger signal to activate macrophages. Strikingly, activation of macrophages by the membrane-associated form of Hsp72 was highly effective than that by the free recombinant Hsp72. This robust effect is likely to be due to the high concentration of Hsp72 within the vesicle (exosome).

Taken together, the results of these studies suggest that the active release hypothesis is an important mechanism by which Hsp72 is released into the extracellular milieu.

Extracellular HSPs act as endogenous danger signals

The 'danger theory' postulates that the host releases endogenous signals that are derived from stressed or damaged cells, capable of stimulating immunity [36]. Accumulating evidence indicates that extracellular HSPs fulfil the criteria of an endogenous danger signal.

HSPs are chaperokines

Preparations of HSPs such as Hsp60, Hsp70, Hsp90, gp96 and Hsp110 purified from a variety of sources including bacterial and mammalians, as well as recombinant bacterial and human products, have been reported to be potent activators of innate immunity, indicating that HSPs act as danger signals. Specifically, these HSP preparations have been reported to stimulate the production of proinflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-12 and the release of nitric oxide (NO) and C-C chemokines by monocytes, macrophages and DCs [10,37–40]. HSPs have also been reported to induce the maturation of DCs, as demonstrated by the upregulation of MHC class I and II molecules and costimulatory molecules such as CD80, CD86 and CD40 [40,41]. Moreover, Chen *et al.* have demonstrated that heat stress induced the release of various HSP from tumor cells, which, in turn, activated tumor cells to produce chemokines for chemoattraction of DCs and T cells via the TLR4 signaling pathway [42]. Thus, it has been proposed that, through their cytokine-like functions, HSPs serve as 'chaperokines' to the host's immune system at sites of tissue injury or stress where HSPs are released into extracellular spaces. The discovery of extracellular biological actions of HSPs as chaperokines is very attractive and the chaperokine theory is interesting with regard to extracellular HSPs as a cause of sterile inflammation.

HSPs augment the action of associated microbial products

There is some concern that these chaperokine activities might have been due to microbial contamination of HSP preparations. The representative microbial product LPS is a strong stimulus of innate immune signaling. Wallin *et al.* reported that highly purified murine liver Hsp70 had no cytokine effects even at concentrations as high as 200–300 mg/ml [43]. Thus, some HSPs have been shown to bind microbial products very efficiently and thus augment the biological actions of microbial products. Habich *et al.* demonstrated that Hsp60 bound LPS tightly and that Hsp60-bound LPS, but not Hsp60 itself, was responsible for the observed cytokine effects of Hsp60 preparation [44]. More interestingly, Hsp60-bound LPS was more potent than LPS in inducing cytokine production. Reed *et al.* showed that gp96 binds endotoxin in a high affinity, saturable and specific manner. The same study demonstrated that low (<0.27 EU endotoxin/mg protein) endotoxin preparations of gp96 do not stimulate NF-κB activation or NO release in macrophages [45]. In addition, Wager *et al.* showed that gp96 bound lipid-based TLR ligands such as palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam_3Cys; a TLR2 ligand) and LPS (TLR4 ligand). Binding of Pam_3Cys and LPS to gp96 enhanced their ability to induce the activation and maturation of DCs [46]. Thus, there may be cooperation between gp96 and TLR ligands, and the former may amplify the effects of the latter. These results suggested that if 'chaperoned by a certain HSP', LPS at very low concentrations could induce cytokine production. In this context, HSP augments immune responses against molecules chaperoned by HSPs but not against HSPs themselves. In a clinical setting, this unique character of HSPs may contribute to the *in vivo* recognition of Gram-negative bacterial infection by binding to LPS and augmenting the host defense system via TLR4. Although further studies are necessary to determine whether the reported cytokine-producing effect is a result of HSPs or contamination of TLR ligands, there is no doubt that HSPs play important roles in eliciting immune responses by acting as danger signals via HSPs themselves and/or HSP-associated molecules.

Cell surface-expressed HSPs elicit immune responses

To avoid the problem of microbial contamination, a number of studies have been conducted on transgenic expression of HSPs on the cell

surface. It has been demonstrated that cell-surface HSPs can activate immune responses. Under these conditions, contamination by exogenous molecules such as LPS is unlikely. Zheng *et al.* demonstrated that cell surface expression of gp96 on tumor cells induced efficient T-cell priming and tumor rejection *in vivo* [47]. Likewise, Chen *et al.* showed that transgenic expression of Hsp70 on the tumor cell surface elicited antitumor immunity, and immunization of mice with these tumor cells led to induction of tumor-specific cytotoxic T cells [38]. In addition, transgenic expression of gp96 on the cell surface leads to *in vivo* activation of DCs and the development of lupus-like systemic autoimmune disease in mice [48,49]. Vaccination of mice with gp96-secreting fibroblasts leads to *in vivo* activation of $CD11b^+$ and $CD11c^+$ APCs [50]. These studies have demonstrated that cell-surface HSPs are capable of activating the immune system *in vivo*. However, it remains unclear whether the observed *in vivo* effects are direct effects of HSPs themselves or effects via HSP-associated molecules.

Getting a grip on the chaperokine world

It must be emphasized that HSP purification procedures should be carried out to the highest standards of molecular chaperone purification as described by Pockley *et al.* [51]. In addition, a further direction of this field will be to identify the receptor binding domain of each HSP. More importantly, x-ray crystallographic analysis may reveal the mode of HSP-receptor binding. From the biological point of view, as an endogenous danger signal, under the condition of bacterial or viral infection, damaged cell-derived extracellular HSPs may bind microbial products such as LPS, lipoproteins, bacterial DNA and viral RNA in the inflamed milieu, leading to activation of TLR- and non-TLR-mediated innate immune responses. This nascent but very attractive field of biology should be further developed through careful experiments.

Endogenous danger signals & sterile inflammation concomitant with immune responses

In addition to recognizing pathogen-associated molecular patterns, the immune system has evolved to recognize endogenous danger signals, called damage-associated molecular patterns (DAMPs), many of which are released by dying or necrotic cells and contribute to 'sterile inflammation' in a noninfectious sterile setting. Furthermore, the recognition of DAMPs can activate the innate immune system *in vivo*. DAMP molecules comprise a structurally and sequence-diverse family of endogenous molecules, generally intracellular and hidden by the plasma membrane, that are often released from necrotic cells. It has been demonstrated that uric acid [22], DNA and more specifically unmethylated CpG-rich DNA regions, high-mobility group box 1 (HMGB1) [52,53], Sin3A-associated protein 130 [54], IL-1α [55], IL-33 [55,56], S100 proteins [57] and HSPs act as DAMPs [58]. However, a recent study has suggested that highly purified HMGB1 does not induce significant amounts of proinflammatory cytokines. The capacity of HMGB1 to bind other molecules may be the underlying basis for these observations [59]. Thus, some of these DAMPs have the ability to interact with other molecules, including DNA, RNA, IL-1β and LPS, and also nucleosomes that augment or modify the function of DAMPs themselves. As mentioned previously, HSPs can also bind a number of endogenous as well as pathogen-associated exogenous molecules and enhance the cytokine effects of these molecules. Recently, we have shown that extracellular Hsp90 can enhance the self-DNA-mediated type I IFN production by plasmacytoid DCs (pDCs) and conventional DCs (cDCs). In the next section we will discuss how Hsp90 is able to change the destination of associated molecules via spatiotemporal regulation.

Hsp90-mediated spatio-temporal regulation of CpG-oligodeoxynucleotide & activation of innate immunity

In contrast to the idea that HSP itself acts as an endogenous danger signal, we have a working hypothesis that HSP empowers the chaperoned innate ligands to activate innate immune response [60]. Unmethylated CpG dinucleotides within certain sequence contexts (CpG motifs) are recognized by TLR9, which is expressed primarily by pDCs and B cells, resulting in a large amount of IFN-α production [61,62]. We have shown that murine and human pDCs pulsed with an Hsp90-CpG-A oligodeoxynucleotide complex produce a larger amount of IFN-α than that in the case of CpG-A alone (FIGURE 1A). Furthermore, unlike human DCs, murine cDCs express both TLR7 and TLR9, although the expression levels are low compared with those in pDCs. We then showed the ability of Hsp90 to target chaperoned CpG-A to murine cDCs, resulting in IFN-α production (FIGURE 1B). The

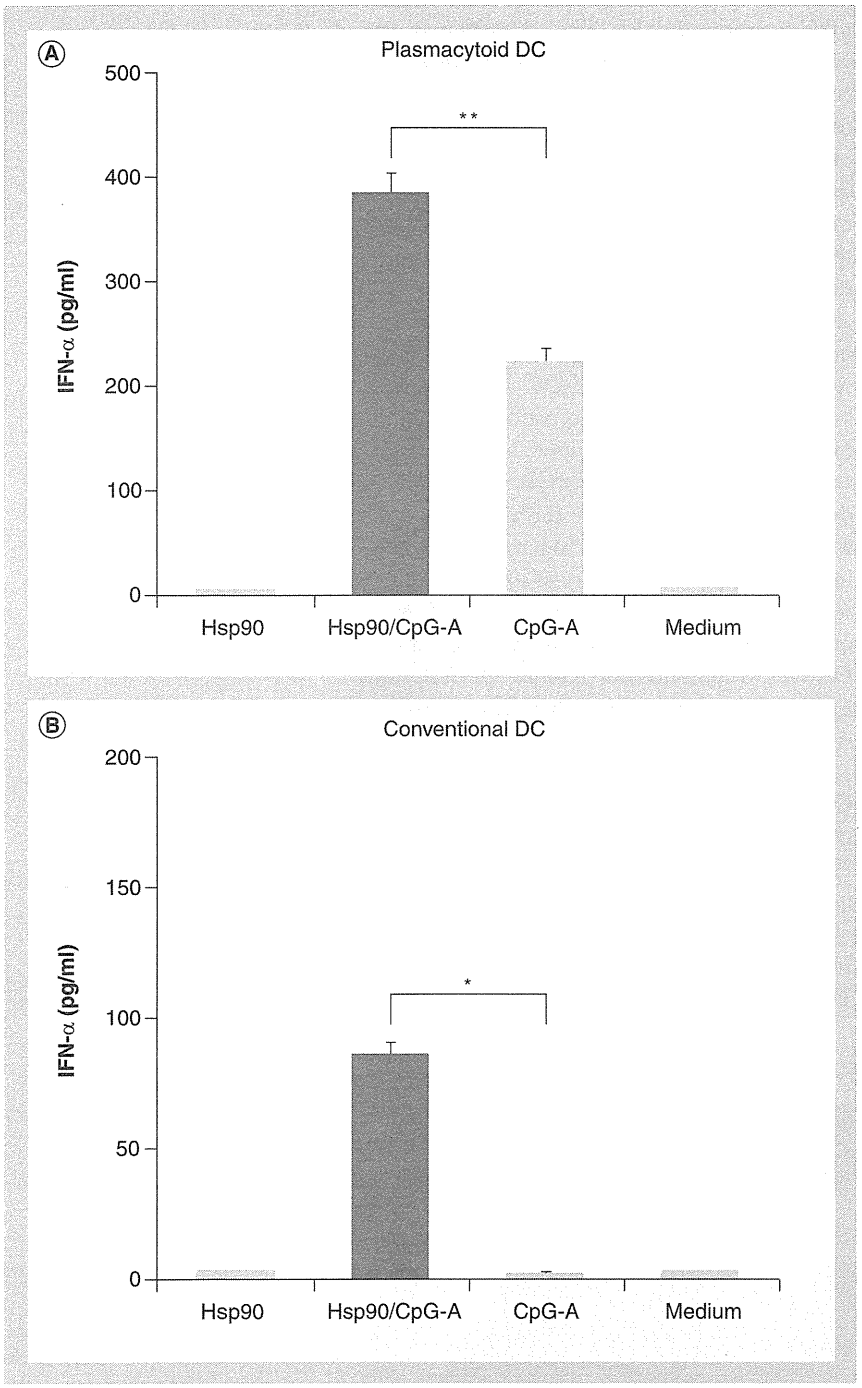


Figure 1. Hsp90–CpG-A complexes enhance induction of IFN-α by murine dendritic cells. (A) Hsp90–CpG-A complexes induce IFN-α production by plasmacytoid DCs to a level twofold higher than that induced by CpG-A alone. **(B)** Conventional DCs produce IFN-α when stimulated with Hsp90-CpG-A complex but not when stimulated with CpG-A alone. Hsp90 alone does not have any effect on IFN-α production. Data are presented as means + standard error of the mean of triplicate wells.
*$p < 0.0005$, **$p < 0.001$; paired student t-test.
DC: Dentric cell.

observed IFN-α production was shown to be TLR9-dependent. We found that Hsp90-chaperoned CpG-A was localized and retained within static early endosomes for longer periods in cDCs, thereby eliciting TLR9 signaling for IFN-α production, but not inflammatory cytokines such as IL-6 and TNF-α. By contrast, CpG-A alone moved into late endosomes and lysosomes within cDCs. Interestingly, not only CpG-A but also CpG-B could stimulate the TLR9 signaling within static early endosomes, resulting in the production of IFN-α. Thus, extracellular Hsp90 has the ability to direct associated CpGs into static early endosomes, leading to interferon regulatory factor 7 activation and IFN-α production.

Why, however, are DNA–Hsp90 complexes retained in early endosomes but not in late endosomes or lysosomes in DCs? We found that endocytosed CpG-A–Hsp90 complexes were selectively transferred into $Rab5^+$, early endosomal antigen (EEA)-1^+ static early endosomes. Very recently, Lakadamyali *et al.* have shown that early endosomes are comprised of two distinct populations, a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome and a static population that matures much more slowly [63]. Cargos destined for degradation, including low-density lipoprotein, EGF and influenza virus, are internalized and targeted to the $Rab5^+$, $EEA1^-$ dynamic population of early endosomes, thereafter trafficking to $Rab7^+$ late endosomes. By contrast, the recycling transferrin is delivered to $Rab5^+$, $EEA1^+$ static early endosomes, followed by translocation to $Rab11^+$ recycling endosomes. They also found that cargos trafficked into these static early endosomes were retained for longer periods and not translocated into late endosomes and lysosomes. Thus, our observation that the CpG-A–Hsp90 complex was retained in static early endosomes, leading to sustained activation of DCs and IFN-α production, is consistent with their findings (Figure 2).

Hsp90 as a possible accelerator for autoimmune diseases

pDCs normally do not respond to self-DNA, which may reflect the fact that viral/bacterial DNA sequences contain multiple CpG nucleotides that bind and activate TLR9, whereas mammalian self-DNA contains fewer such motifs, which are most likely masked by methylation. Recent evidence, however, suggests that self-DNA has the potential to trigger TLR9 but may fail to do so because it fails to access the TLR9-containing endolysosomal compartments. One of the mechanisms to which this effect is attributed is the fact that DNase easily and rapidly breaks down extracellular DNA, thereby hampering self-DNA localization into

endocytic compartments. The importance of this mechanism in preventing autoimmune responses has been shown by the fact that mice deficient in DNase II develop systemic lupus erythematosus (SLE)-like syndrome [64]. Recently, it has been demonstrated that pDCs do sense and respond to self-DNA in human autoimmune diseases. Therefore, we examined whether Hsp90 targets self-DNA into static early endosomes, resulting in IFN-α production by human pDCs [60]. Upon Hsp90-mediated enforced endosomal translocation, both human self-DNA and CpG-ODN could activate DCs via TLR9 to produce IFN-α. Previous studies have demonstrated the presence of autoantibodies to Hsp90 [65,66] and enhanced expression of Hsp90 in peripheral blood mononuclear cells of patients with active SLE [67,68], suggesting a role of Hsp90 in the pathogenesis. In addition, Hsp90 has been shown to localize both in the cytoplasm and nucleus [69]. Moreover, under stressful conditions, it has been shown that cytosolic Hsp90 translocates to the nucleus [70]. This suggests that Hsp90 may bind self-DNA within the nucleus. When cells undergo necrosis, self-DNA associated with endogenous Hsp90 could be released into the extracellular space and might trigger IFN-α production by pDCs. Our findings support the idea that Hsp90, an endogenous danger signal molecule found in sera of SLE patients, might be the key mediator of pDC activation in SLE. Thus, Hsp90 may activate innate immunity to self-DNA by forming a complex with self-DNA that is delivered to and retained within early endocytic compartments of pDCs to trigger TLR9 and induce IFN production. Thus, we determined a fundamental mechanism by which pDCs sense and respond to self-DNA coupled with Hsp90. Our data suggest that, through this pathway, pDCs drive autoimmunity in autoimmune diseases. Several host factors other than Hsp90 that can convert self-DNA into a trigger of DC activation have been reported. Endogenous cationic antimicrobial peptide LL37 (also known as CAMP) [71], autoantibodies [72] and HMGB1 [59] have been demonstrated to do so by forming a complex with host-derived DNA. Together, these findings indicate that the ability of some of DAMPs to convert self-DNA into a trigger of high levels of IFN-α production depends on the capacity of DAMPs to concentrate and retain DNA in static early endosomes, thus enabling the selective and sustained activation of early endosomal TLR9.

Cross-presentation mediated by HSP: pivotal role of Hsp90

HSPs inherently act as molecular chaperones, and in our opinion, from an immunological point of view, the most characteristic finding is that HSPs can bind antigenic peptides within cells. Therefore, immunization with a HSP–antigenic

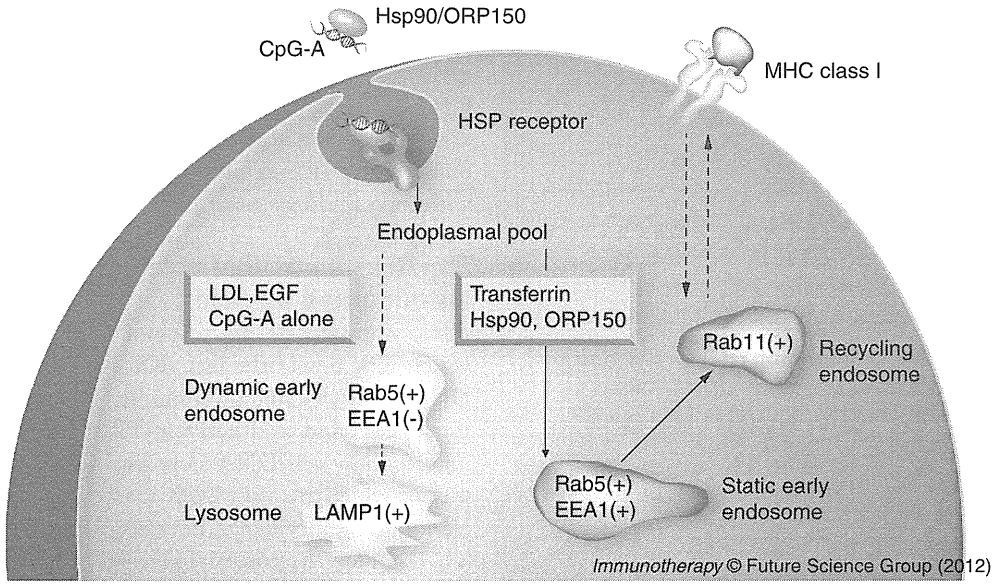


Figure 2. Extracellular Hsp90/ORP150 targets chaperoned molecules into static early endosomes. Early endosomes are comprised of static early endosomes (Rab5$^+$ and EEA-1$^+$) and dynamic early endosomes (Rab5$^+$ and EEA-1$^-$). Hsp90/ORP150-antigen or Hsp90–CpG-A complexes are preferentially targeted and retained in static early endosomes of DCs, leading to efficient antigen cross-presentation as well as sustained activation of Toll-like receptor 9 and IFN-α production.
Hsp: Heat-shock protein; LDL: Low-density lipoprotein.

peptide complex elicits antigen-specific cytotoxic T-cell responses. Therefore, extracellular HSP can deliver associated antigens into the MHC class I presentation pathway of antigen-presenting cells, a process called cross-presentation, thus inducing antigen-specific $CD8^+$ T-cell responses. However, the underlying mechanism for introduction of an exogenous antigen into a cross-presentation pathway remains unclear, and the precise mechanism for intracellular antigen translocation and the processing pathway have not been fully elucidated. Recent evidence indicates that exogenous antigens can be processed through at least two distinct pathways, one involving access of exogenous antigens to the classical MHC class I loading pathway (TAP-dependent) and the other involving unconventional post-Golgi loading of MHC class I molecules in endocytic compartments (TAP-independent) [73]. One or both of these pathways can contribute to cross-presentation depending on the source of exogenous antigens such as soluble proteins, immune complexes or peptides chaperoned by HSPs.

We first demonstrated that extracellular Hsp90-peptide complexes generated *in vitro* were efficiently cross-presented by DCs and recognized by peptide-specific CTLs via a TAP-independent 'endosome-recycling' pathway because DCs derived from TAP-knockout mice were fully functional in cross-presentation of an Hsp90–peptide complex [74]. Next, we showed that cross-presentation of extracellular Hsp90–ovalbumin (OVA) protein complexes to specific $CD8^+$ T cells involved both classical proteasome–TAP-dependent and TAP-independent endosome-recycling pathways [75]. Using confocal microscopy, we found that the internalized extracellular Hsp90 and OVA colocalized with cytosolic proteasomes. To investigate whether endogenous Hsp90 was responsible for cross-presentation of the exogenous Hsp90–OVA complex, we treated DCs with the Hsp90-specific inhibitor radicicol in a cross-presentation assay. The results showed that treatment of DCs with radicicol did not affect the cross-presentation of exogenous Hsp90-OVA, suggesting that endogenous Hsp90 might not be responsible for the exogenous Hsp90-mediated cross-presentation. Strikingly, when an anti-Hsp90 monoclonal antibody was introduced into DCs, the colocalization of internalized Hsp90-chaperoned OVA and proteasomes was abolished, resulting in inhibition of TAP-dependent cross-presentation of OVA. Thus, extracellular Hsp90 may play a pivotal role in the translocation of chaperoned antigens for proteasomal degradation in the cytosol. By contrast, a high concentration of soluble OVA (200 mg/ml) was cross-presented by DCs, although the cross-presentation was considerably less efficient than that of a Hsp90–OVA complex (20 μg/ml of OVA). Interestingly, this cross-presentation was radicicol-sensitive, indicating that endogenous Hsp90 played an important role in the cross-presentation of exogenous OVA. Udono *et al.* have elegantly demonstrated that endogenous Hsp90α was required for cross-presentation of exogenous OVA using DCs derived from Hsp90α-knockout mice [76]. Their findings also suggest that endogenous Hsp90, as well as exogenous Hsp90, might help the exogenous Hsp90–OVA complex translocate into cytosol at the cytosolic face for cross-presentation (FIGURE 3). By contrast, OVA chaperoned by Hsp90 was not presented by MHC class II molecules *in vitro* or *in vivo*, although the antigen was exogenously loaded onto DCs. To confirm these observations, we investigated the destination of Hsp90–OVA complexes and soluble OVA after its uptake in DCs, using confocal laser microscopy. Hsp90-chaperoned OVA was observed to colocalize with early endosomes, recycling endosomes, and proteasomes but not lysosomes. By contrast, soluble OVA was detected in early endosomes to lysosomes but not in recycling endosomes, ER or proteasomes. These findings indicated that Hsp90–OVA complexes and soluble OVA were sorted into different organelles. Thus, extracellular Hsp90 might be essential for translocation of chaperoned antigens from the extracellular milieu into the cytosol, resulting in proteasomal degradation for cross-presentation. These results have revealed a novel mode of involvement of Hsp90 in antigen presentation by DCs: exogenous Hsp90 preferentially introduces the chaperoned antigen into the MHC class I pathway, resulting in efficient cross-presentation. Since there is a classical paradigm that extracellular antigens are presented by MHC class II molecules, it seemed significant to find that Hsp90 changed the destination of the associated antigen on antigen presentation. Calderwood's group has recently reported that the SREC-1 acts as an Hsp90 receptor for cross-presentation on DCs [77]. As described previously, Lakadamyali *et al.* demonstrated that early endosomes are comprised of two distinct populations: a dynamic population that matures rapidly toward the late endosome and lysosome, and a static early endosome that