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研究成果の刊行物・別刷

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Differentiation, Maturation, and Survival of Dendritic Cells by Osteopontin Regulation

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Dendritic cells (DCs) are antigen-presenting cells with the ability to induce primary immune responses necessary in innate immunity and adaptive immunity. Osteopontin (OPN) is a secreted acidic phosphoprotein containing an arginine-glycine-aspartate sequence and has been suggested to play an important role in early cellular immune responses. The interaction between DCs and OPN has not been clarified. We hypothesized that there is an important interaction between DCs and OPN, which is an indispensable extracellular matrix component in early cellular immune responses. Human monocyte-derived DCs synthesized OPN especially during the differentiation from monocytes to immature DCs. By blocking of OPN with anti-OPN antibody, cultured DCs became smaller and expressed lower levels of costimulatory molecules and major histocompatibility complex class II antigens than untreated DCs. Furthermore, DCs treated with anti-OPN antibody easily underwent apoptosis. These results suggest that human DCs can produce OPN and that OPN may play a role in the differentiation, maturation, and survival of DCs by autocrine and/or paracrine pathways.

Dendritic cells (DCs) play critical roles in innate immunity and adaptive immunity (4). Immature DCs reside in peripheral tissues, where they serve as sentinels for foreign antigens and microbial pathogens. Upon activation, immature DCs undergo maturation and migrate to the lymph nodes. During maturation, DCs acquire an enhanced capacity to form and accumulate peptides, major histocompatibility complex (MHC) class II molecules, costimulatory molecules (such as CD40, CD80, and CD86), and antigens of unknown functions (such as CD83 and DC-LAMP) (10). Mature DCs can prime naïve T cells and initiate primary T-cell-mediated immune responses (4). In addition, there is increasing evidence that DCs in situ induce antigen-specific unresponsiveness or tolerance in central lymphoid organs and in peripheral tissues (4, 31). Thus, DCs play a crucial role during the initiation and regulation of immune responses. Recently, we and others reported that DCs are essential for granuloma formation against bacterial antigens in animal models (12, 33, 36).

Osteopontin (OPN), also known as early T-lymphocyte activation-1 (Eta-1), is a phosphoprotein that contains arginine-glycine-aspartate (RGD). Although OPN is classified as an extracellular matrix (ECM) protein, OPN has only recently been shown to be an important component of early cellular immune responses (18). OPN has various functions in chemotaxis for immune cells, tumor metastasis, neovascularization, and host defense, including control of nitric oxide production, control of infection, and control of cell adhesion (3, 5, 9, 21, 25). These mechanisms are regulated by posttranslational modifications, such as cleavage by thrombin, addition of a glucose chain, and phosphorylation. Various immunological disorders are associated with high levels of OPN expression (8, 15). Analyses of OPN-deficient mice revealed that OPN plays an important immunological role in granuloma formation (23), acid-fast bacillus disease (21), and carcinoma metastasis (5). The role of OPN in inflammation suggests that ECM-related proteins may function as pleiotropic cytokines to regulate immune responses. Activated macrophages, lymphocytes, and natural killer (NK) cells produce OPN in response to various stimuli (23). However, there are no reports of the effects of OPN on DCs, with the exception of a single report of the migratory effect of OPN on cutaneous Langerhans cells and DCs in a mouse allergic cutaneous hypersensitivity model (34). The direct effect of OPN on the development and activation of DCs has not been clarified. Thus, we sought to characterize the functional interaction between OPN and DCs by examining the effects of OPN on differentiation, maturation, and function of human monocyte-derived immature and mature DCs. We report here that human monocyte-derived dendritic cell (Mo-DC) can produce OPN that enhances differentiation, maturation, and survival of DCs by autocrine and/or paracrine pathways.

MATERIALS AND METHODS

Reagents. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was kindly provided by Kirin Brewery (Tokyo, Japan). Recombinant human interleukin-4 (IL-4) was purchased from R&D Systems (Minneapolis, Minn.). Anti-OPN monoclonal antibody (MAb) (mouse immunoglobulin G1 [IgG1]) was from IBL (Gunma, Japan). Phycoerythrin (PE)-conjugated anti-human HLA-DR antibody, Fc receptor, and fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 were from Sigma (St. Louis, Mo.). Anti-human CD86 antibody was purchased from BD PharMingen (San Diego, Calif.), and anti-human CD83 and isotype control IgG were from Immunotech (Marseille, France). Lipopolysaccharide (LPS) (*Escherichia coli*) (catalog no. L4391), β-D-glucan (barley) (catalog no. G6513), and lipoteichoic acid (LTA) (*Staphylococcus aureus*) (catalog no. L2515) were from Sigma.

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Generation of DCs and macrophages from purified human CD14$^+$ monocytes. Mo-DCs were obtained as previously described (29) but with a minor modification. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples from healthy volunteers by standard density gradient centrifugation with Lymphoprep (Axis-Shield, Oslo, Norway). PBMCs at the interface were pelleted and washed twice with phosphate-buffered saline (PBS). CD14$^+$ monocytes were isolated from mononuclear fractions through positive selection with microbeads coated with anti-CD14 antibody and Midi-Macs separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity was checked by flow cytometry with anti-CD14 MAb and was >95%.

For Mo-DC generation, purified CD14$^+$ monocytes were cultured in complete medium (CM), which consisted of RPMI 1640 medium supplemented with $NaHCO_3$, L-glutamine (Nipro, Osaka, Japan), 10% fetal calf serum (FCS), 10 mg of streptomycin per ml, 10,000 U of penicillin G per ml, 55 mM 2-mercaptoethanol, and HEPES, at a concentration of 5×10^5 or 2.5×10^5 cells/ml in 24-well flat-bottom microplates (Becton Dickinson, Franklin Lakes, N.J.). GM-CSF (800 U/ml) and IL-4 (500 U/ml) were added to the CM to generate Mo-DCs. Cells were incubated at 37°C in a 5% CO_2 atmosphere. On day 5, the cultured cells progressed to immature DCs as confirmed by fluorescence-activated cell sorting (FACS) analysis of surface markers and by morphology. To generate mature DCs, immature DCs were harvested on day 5, washed with PBS, and seeded (5×10^5 cells/ml/well) in fresh CM supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml), and stimulated with LPS (1 μg/ml), β-D-glucan (5 μg/ml), LTA (20 μg/ml), or CD40L (1 μg/ml) for 48 h.

Monocyte-derived macrophages (Mo-Mϕs) were generated as described previously (16). CD14$^+$ monocytes were cultured (37°C, 5% CO_2) in CM supplemented with M-CSF (10^4 U/ml) for 5 days. During culture, monocytes underwent morphological changes characteristic of macrophages differentiated from monocytes, such as an increasing size and adherence. Purity was >95% as verified by FACS analysis with an anti-CD14 MAb and an anti-human Fc receptor MAb and by morphology and enhanced phagocytosis of latex particles.

Cytology. DCs generated in vitro were cytocentrifuged for 5 min at 500 × *g* (Cytospin 3; Shandon, Astmoor, United Kingdom) and stained with Diff-Quick (Kokusai Shiyaku, Kobe, Japan). Mature DCs were larger, double the size of monocytes in diameter with long cytoplasmic projections (dendrites), eccentric multilobulate lateral nuclei, and abundant cytoplasm. Immature DCs had small cytoplasmic projections or no projections at all.

Flow cytometry. Cells were washed twice with PBS supplemented with 2% FCS and resuspended in PBS supplemented with 2% FCS. Cells were incubated with Abs at saturating concentrations for 30 min at 4°C and then washed with PBS two more times. Cells were stained with the following Abs: FITC-conjugated anti-CD14 antibody (Sigma), PE-conjugated anti-HLA-DR antibody (Sigma), anti-CD83 antibody (Immunotech), and anti-CD86 antibody (PharMingen). Rabbit FITC-conjugated anti-mouse immunoglobulin (DakoCytomation, Kyoto, Japan) was used as a secondary antibody. Cell surface antigen expression was evaluated by single- or double-immunofluorescence staining, and analysis was performed with a FACScan analyzer and CellQuest software (Becton Dickinson).

Measurement of OPN. The concentration of OPN in PBMC culture supernatants was measured with a human OPN enzyme immunoassay (EIA) kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) according to the manufacturer's instructions.

Detection of apoptosis. Apoptosis was detected by staining with an annexin V-FITC kit (Immunotech) according to the manufacturer's protocol. Cells were harvested, washed twice with PBS, and labeled with annexin V-FITC and propidium iodide (PI) for 10 min on ice. Annexin V and PI staining was examined with a FACScan analyzer and CellQuest software (Becton Dickinson).

Statistical analysis. Student's paired *t* test was used to determine the significance of different mean values, and a *P* value of <0.05 was taken to indicate statistical significance.

RESULTS

Production of OPN by human monocytes and immature DCs. A previous study revealed that OPN is produced by activated macrophages and T cells and that expression of OPN is induced by GM-CSF signaling in hematopoietic cells (17). We hypothesized that Mo-DCs produce OPN and that there is some functional interaction between OPN and GM-CSF, a cytokine necessary for generation of DCs. We analyzed changes in OPN production by Mo-DCs during maturation from monocytes to DCs. Immature Mo-DCs were generated by culturing CD14$^+$ monocytes (5×10^5 cells/ml/well) in CM supplemented with GM-CSF (800 U/ml) and IL-4 (500 U/ml) for 5 days. Monocytes cultured in CM without GM-CSF and IL-4 for 5 days were used as a control. Control cells showed no morphological changes and still expressed CD14 antigen after culture (data not shown). In contrast, immature Mo-DCs lost CD14 surface antigen and were twice as large as monocytes in diameter, as previously described (30).

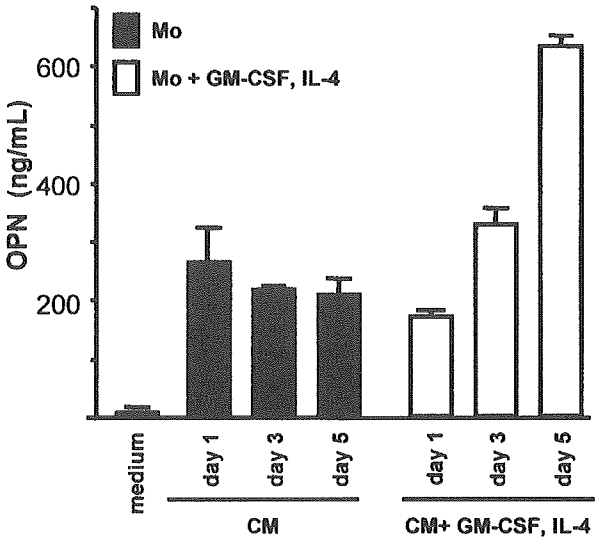

FIG. 1. OPN production by human monocytes and immature DCs. Human CD14$^+$ monocytes (Mo) were incubated for 5 days in the presence and absence of GM-CSF (800 U/ml) and IL-4 (500 U/ml). Twenty-four, 72, and 120 h after incubation, OPN in the culture supernatant was quantified with an EIA kit. OPN production by monocyte-derived maturing DCs increased during the culture period, whereas production by monocytes did not change. Data are shown as the means ± standard errors of the means (error bars) from three independent experiments.

As shown in Fig. 1, monocytes and cells maturing to immature DCs produced OPN. OPN was detected at levels as high as 200 ng/ml in culture supernatants of monocytes cultured in the absence of GM-CSF and IL-4. However, a meaningful increase in OPN production was not observed during the culture period. In contrast, for immature DCs incubated with GM-CSF and IL-4, a significant increase in OPN production during the culture period was observed. The supernatants on day 5 contained more than 600 ng of OPN per ml, which is triple that of the monocytes. This suggests that human monocytes can produce OPN and that OPN production is enhanced by maturation to immature DCs.

OPN production by immature DCs decreased during their maturation. Immature Mo-DCs obtained by 5-day culture of CD14$^+$ monocytes with GM-CSF (800 U/ml) and IL-4 (500 U/ml) were collected, washed with PBS, and cultured for another 48 h with or without stimulants, such as LPS (1 μg/ml), LTA (20 μg/ml), and β-D-glucan (5 μg/ml). Pathogen-associated molecular patterns (PAMPs), such as LPS and LTA, stimulate maturation of DCs. This leads to enhanced antigen processing, increased MHC class II expression, and induction of costimulatory molecules, such as CD80 and CD86.

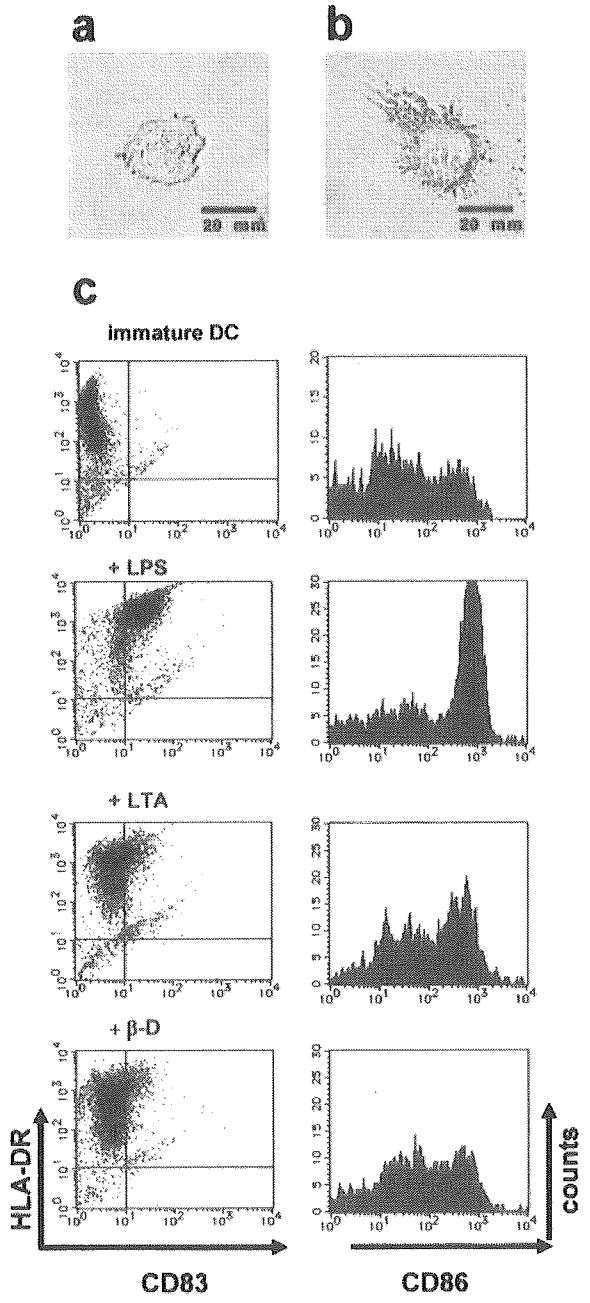

FIG. 2. Changes in morphology and immunophenotype of immature and mature monocyte-derived DCs. (a) Immature DCs. After magnetic cell sorting, $CD14^+$ monocytes that were incubated in CM with GM-CSF (800 U/ml) and IL-4 (500 U/ml) for 5 days acquired the morphological characteristics of immature DCs (magnification, ×200). (b) Mature DCs. Immature DCs were stimulated with LPS (1 μg/ml) for 48 h to generate mature DCs. LPS induced maturation of DCs with characteristic dendrites (magnification, ×200). (c) Flow cytometric analysis of CD86, HLA-DR, and CD83 on immature DCs and mature DCs treated with PAMPs. Immature DCs were stimulated with various PAMPs and harvested, and expression of CD83, CD86, and HLA-DR antigens was examined by flow cytometry. DCs stimulated with LPS (1 μg/ml) showed increased expression of CD83, CD86, and HLA-DR. In comparison, LTA (20 μg/ml) and β-D-glucan (β-D) (5 μg/ml) also triggered DC maturation, although they did not lead to full maturation. The results shown are from a single experiment using cells from a single donor and are representative of three independent experiments that gave similar results.

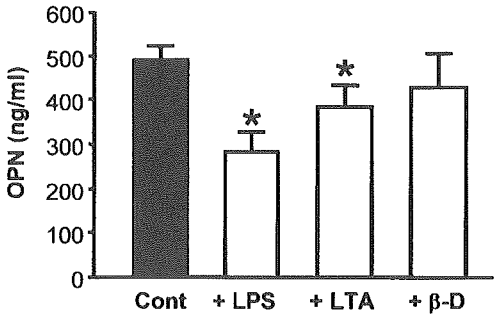

FIG. 3. OPN production by immature DCs and mature DCs treated with PAMPs. Immature DCs were stimulated with LPS (1 μg/ml), LTA (20 μg/ml), or β-D-glucan (β-D) (5 μg/ml) for 48 h to generate mature DCs or left alone as a control (Cont). Maturation of DCs was confirmed by morphology and expression of surface markers (Fig. 2c). OPN production by immature and mature DCs was measured with an EIA kit. Immature human DCs synthesized OPN, and production decreased during maturation. Production of OPN by LPS-treated mature DCs decreased to half that of immature DCs. LTA-stimulated mature DCs also produced less OPN than immature DCs. β-D-glucan-stimulated DCs showed a tendency to produce less OPN, but the difference was not statistically significant. The results shown are means ± standard errors of the means (error bars) from a single experiment using cells from a single donor and are representative of three experiments that gave similar results. Values that were significantly different ($P < 0.05$) from the control value (asterisk) are indicated.

As shown in Fig. 2c, LPS (1 μg/ml) and LTA (20 μg/ml) enhanced surface expression of CD83, CD86, and HLA-DR, although β-D-glucan (5 μg/ml) did not up-regulate expression of costimulatory molecules and HLA-DR as strongly as LPS did. OPN levels in culture supernatants were measured by EIA. Human Mo-DCs that matured in response to stimulation by LPS (1 μg/ml) or LTA (20 μg/ml) produced significantly less OPN than immature DCs did ($P < 0.05$) (Fig. 3). Analysis of surface markers of immature DCs stimulated with β-D-glucan (5 μg/ml) revealed that the maturation signal in our study system was insufficient to obtain fully mature DCs (Fig. 2c), and the significant decrease in OPN production that occurs with DC maturation was not observed (Fig. 3).

Maturation of DCs was confirmed by analysis of surface expression of CD83 and HLA-DR and by morphology. These results suggest that OPN production decreases when DCs mature fully. To confirm this, we used 1 μg of CD40L per ml as a nonpathogenic stimulant to obtain fully mature DCs. Immature DCs were incubated for 48 h in CM with 1 μg of CD40L per ml. OPN was produced in CD40L-treated mature DCs; however, the mature DCs produced significantly less OPN than the nonstimulated immature DCs (Fig. 4a). These results suggest that OPN production decreases when DCs mature fully in response to various stimulants.

Activated macrophages are known to produce OPN (23). We generated monocyte-derived macrophages, and activated macrophages were obtained by stimulation with LPS (1 μg/ml) and CD40L (1 μg/ml) as described above for activated DCs. OPN production increased significantly in activated macrophages (Fig. 4b). These results suggest that OPN production during maturation or activation is regulated differently in DCs and macrophages.

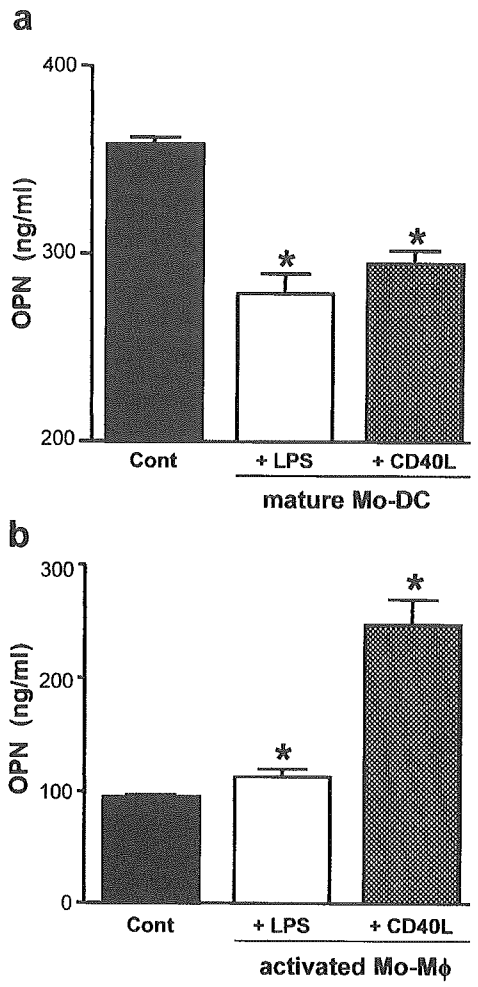

FIG. 4. Comparison of OPN production by mature DCs and activated macrophages. (a) OPN production by LPS- or CD40L-stimulated mature DCs. Immature DCs (5×10^6 cells/ml/well) were incubated with LPS (1 μg/ml) or CD40L (1 μg/ml) to obtain mature monocyte-derived DC (Mo-DC). LPS- or CD40L-treated DCs acquired the morphology characteristic of mature DCs and showed increased expression of HLA-DR, CD86, and CD83 (data not shown). Both LPS- and CD40L-induced mature DCs produced OPN. However, production of OPN by LPS- and CD40L-induced mature DCs was lower than that induced by control (Cont) immature DCs. Data shown are the means ± standard errors of the means (error bars) from three independent experiments. (b) OPN production by LPS- or CD40L-stimulated monocyte-derived macrophages (Mo-Mϕ). To obtain Mo-Mϕ, human monocytes were incubated with M-CSF (10^4 U/ml) for 120 h. After incubation, these cells were CD14 positive and had a great deal of cytoplasm (data not shown). Activated Mo-Mϕ were obtained by stimulation with LPS (1 μg/ml) or CD40L (1 μg/ml). After 48 h, the supernatant was analyzed for OPN levels. Activated Mo-Mϕ, especially CD40L-stimulated Mo-Mϕ, synthesized more OPN than did unstimulated Mo-Mϕ. Results shown are the means ± standard errors of the means (error bars) from three independent experiments. Values that were significantly different ($P < 0.05$) from the control value (asterisk) are indicated.

OPN may be involved in DC viability and differentiation from monocytes. In this study, we observed production of OPN by human monocytes and Mo-DCs. Because OPN production was increased during differentiation from monocytes to immature DCs, we hypothesized that OPN enhances differentiation and maturation of Mo-DC, especially during the early stage of differentiation. To examine the function of OPN, purified $CD14^+$ monocytes were cultured with GM-CSF (800 U/ml) and IL-4 (500 U/ml) with or without a neutralizing OPN MAb at concentrations of 1, 3, and 5 μg/ml. Mo-DCs from cultures with or without anti-OPN Ab lost CD14 surface antigen, indicating that the cells had differentiated from monocytes to immature DCs. Looking at the morphology of the cells, the cells appeared to have differentiated into immature DC-like cells even when cultured with anti-OPN Ab. Immature DCs treated with 3 or 5 μg of anti-OPN Ab per ml were smaller than control immature DCs; however, they still possessed morphological features characteristic of immature DCs and differed in appearance from the 5-day culture of monocytes used as a control (Fig. 5a and b). There were no morphological differences between immature DCs treated with anti-OPN Ab (1 μg/ml) and untreated immature DCs. During incubation with or without anti-OPN Ab, cell viability was maintained at over 80%. We then analyzed expression of HLA-DR, CD83, and CD86 by immature DCs treated with anti-OPN Ab or not treated with the Ab. As expected, immature DCs did not express CD83, which is a reliable marker of DC maturation. Surface expression of CD86 and HLA-DR was lower in immature DCs treated with anti-OPN Ab than in untreated control immature DCs (Fig. 5c).

To examine whether OPN mediates maturation of immature DCs to mature DCs, final maturation was induced by stimulation with LPS (1 μg/ml) for 2 days in the presence or absence of anti-OPN Ab (Fig. 6). Maturation and activation were evaluated on the basis of morphology and expression of HLA-DR, CD83, and CD86. LPS stimulation without anti-OPN Ab caused immature DCs to mature (Fig. 6a). Stimulation in the presence of anti-OPN Ab caused immature DCs to develop poor dendrites and little cytoplasm (Fig. 6b). HLA-DR and CD86 expression was lower in maturing DCs not treated with anti-OPN Ab than in mature control DCs (Fig. 6c). CD83 expression was higher in all viable mature DCs than in immature DCs, and there was no significant difference in the mean fluorescence level of CD83 in control untreated DCs and DCs treated with anti-OPN Ab. These results suggest that final maturation and activation as judged by increased expression of CD86 and HLA-DR were inhibited by anti-OPN Ab. Even 1 μg of anti-OPN Ab per ml showed a sufficient inhibitory effect. Furthermore, DCs exposed to 5 μg of anti-OPN Ab per ml contracted, and more than 50% of the cells underwent apoptosis.

Effects of OPN on viability and survival of DCs. Several reports have suggested that OPN may play a role in cell survival. OPN has been shown to inhibit apoptosis in smooth muscle cells, endothelial cells (14), epithelial cells (22), and pro-B cells (28). In one study, LPS stimulation caused DCs treated with anti-OPN Ab (5 μg/ml) to contract, and cell recovery was low. However, the roles of OPN in regulation of survival and death of DCs have not been elucidated. We attempted to determine whether OPN is involved in the viability of DCs with annexin V and PI staining. The effects of anti-OPN Ab on survival of monocytes, immature DCs, and LPS-stimulated mature DCs were examined. Annexin V staining revealed that anti-OPN Ab at concentrations of 1 and 5 μg/ml significantly reduced the viability of monocytes from more than

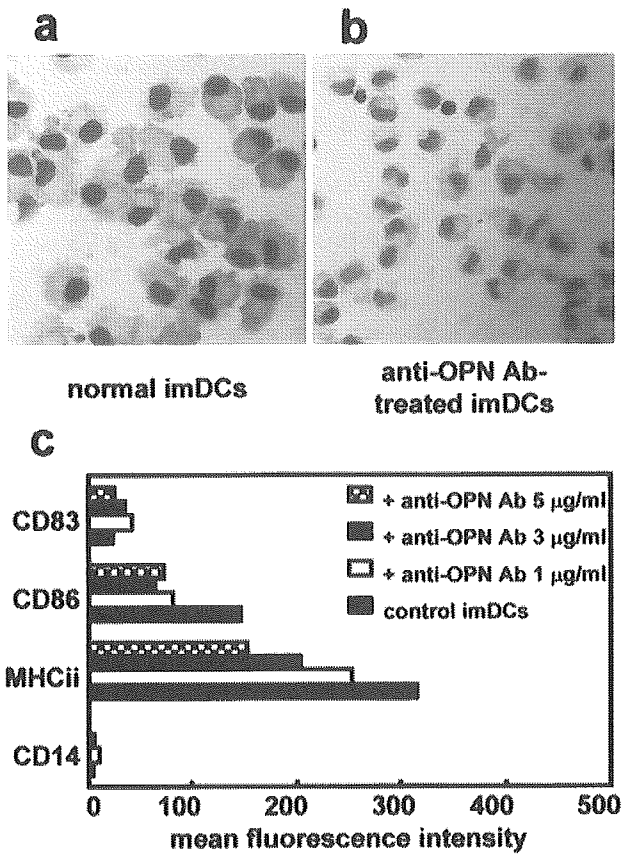

FIG. 5. OPN mediates differentiation from monocytes to immature DCs. To obtain immature DCs and immature DCs treated with anti-OPN antibody, monocytes were cultured with GM-CSF (800 U/ml) and IL-4 (500 U/ml) in the presence or absence of anti-OPN Ab (1, 3, or 5 μg/ml) for 120 h. Cells were collected, cytocentrifuged, stained with Diff-Quick, and morphological changes were observed by light microscopy. Surface expression of HLA-DR (MHCii), CD83, and CD86 on DCs generated in the presence of anti-OPN Ab (1, 3, or 5 μg/ml) were analyzed by flow cytometry. (a) Morphology of immature DCs (imDCs) (Diff-Quick staining) (original magnification, ×400). (b) Morphology of immature DCs generated with 5 μg of anti-OPN Ab per ml (Diff-Quick staining) (original magnification, ×400). Immature DCs treated with anti-OPN Ab were smaller than control immature DCs. (c) Surface expression of HLA-DR, CD83, and CD86 on immature DCs and immature DCs treated with anti-OPN Ab. Immature DCs treated with anti-OPN Ab produced lower levels of HLA-DR and CD86 than control immature DCs. The inhibitory effect of anti-OPN Ab on DC differentiation occurred in a dose-dependent manner. Results are representative of three independent experiments.

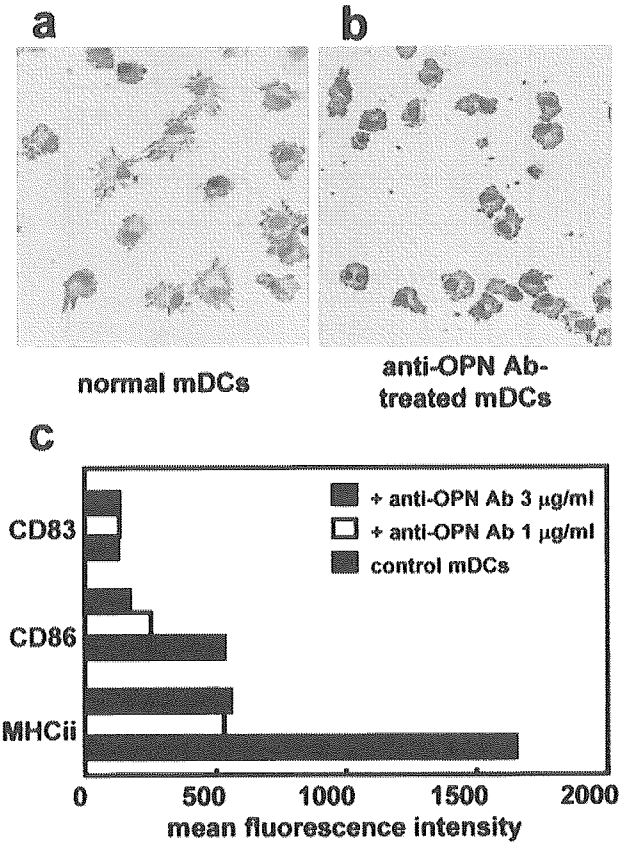

FIG. 6. OPN mediates maturation from immature DCs to mature DCs. To induce DC maturation, immature DCs were incubated with LPS (1 μg/ml) for another 48 h with or without anti-OPN Ab (1, 3, or 5 μg/ml). (a) Control mature DCs (mDCs) obtained by stimulation with LPS (1 μg/ml) acquired the morphology characteristic of DCs. (b) Maturing DCs treated with anti-OPN (1 μg/ml) Ab also showed dendritic morphology. However, the majority of cells remained in the immature state as judged by morphology. (c) Changes in surface expression of HLA-DR (MHCii), CD83, and CD86 on DCs after maturation. Mature DCs expressed high levels of MHC class II and CD86, whereas maturing DCs treated with anti-OPN Ab expressed lower levels of HLA-DR and CD86, whereas the inhibitory effect of anti-OPN Ab on DC maturation was dose dependent. Results are representative of three independent experiments.

90 to 80% (data not shown). As shown in Fig. 7, immature DCs treated with anti-OPN Ab (1 and 5 μg/ml) underwent apoptosis at a rate of 15 to 20%. More than 50% of mature DCs treated with a high dose of anti-OPN Ab and LPS underwent apoptosis; only 16.7% of cells underwent apoptosis at an Ab concentration of 1 μg/ml. These results suggest that OPN is necessary for DC maturation and survival.

DISCUSSION

OPN is a phosphorylated acidic glycoprotein that is expressed in a variety of tissues as a component of the ECM. Expression of OPN by many types of cells, including macrophages, T cells, NK cells, endothelial cells, smooth muscle cells, and epithelial cells, has been reported (7, 24, 26). Several studies have suggested that OPN plays a role in regulation of inflammatory cell accumulation at sites of inflammation and repair (23). OPN expression has been reported under both physiological and pathological conditions, such as tuberculosis (20) and sarcoidosis (24). OPN-knockout mice develop inadequate antimicrobial immunity to a broad range of pathogens. One study revealed that these mice have defects in their ability to clear *Listeria monocytogenes* after systemic infection (3), and another showed that these mice have increased susceptibility to infection by mycobacteria (21). O'Regan and colleagues (25) reported abnormal granuloma formation in the lungs of OPN-deficient mice. Furthermore, OPN expression in humans also contributes to resistance to mycobacterial infection (19). Therefore, OPN not only participates in the maintenance or

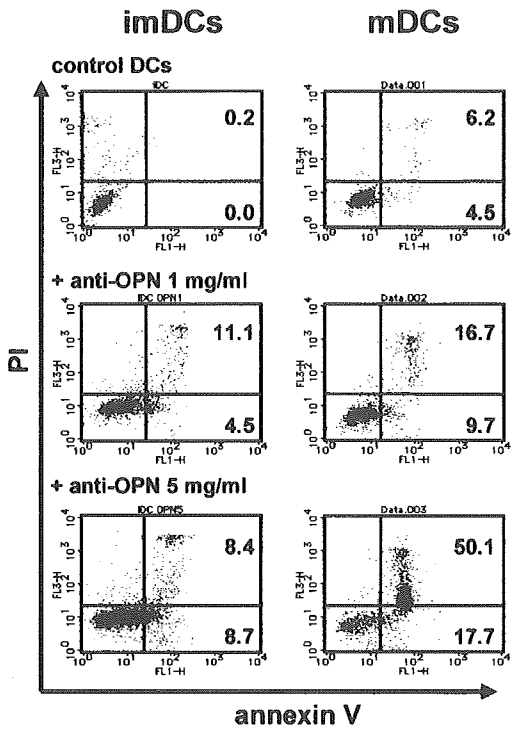

FIG. 7. Effect of OPN on apoptosis of DCs. Immature DCs (imDCs) generated from monocytes incubated in the presence or absence of anti-OPN Ab (1, 3, or 5 μg/ml) were collected on day 5 and analyzed for apoptosis by annexin V and PI staining. Anti-OPN Ab treatment increased apoptosis of immature DCs from 0.2% of immature control DCs to 8.4% of immature DCs treated with Ab (5 μg/ml). Immature DCs were collected and resuspended in new CM in the presence or absence of anti-OPN Ab and stimulated with LPS (1 μg/ml) for 48 h to obtain mature DCs (mDCs), and then mature DCs were collected and analyzed for apoptosis by annexin V and PI staining. Approximately 6.2% of induced mature DCs induced by LPS stimulation underwent apoptosis. Anti-OPN Ab treatment raised the rate of apoptosis to 50.1% in a dose-dependent manner. The results shown were obtained from a single experiment and are representative of three independent experiments that gave similar results.

reconfiguration of tissue integrity during inflammatory processes but also plays a role in cell-mediated immunity. However, the precise role of OPN in immune responses is still unclear.

DCs play a crucial role during the initiation and regulation of immune responses. They are essential for the containment of infections that induce cellular immune responses (12, 27, 33, 36). We recently reported that DCs play a key role in the initiation of cell-mediated immune granuloma formation (12).

Recently, Ahn and colleagues (1) reported that monocyte-derived DCs express the *OPN* gene. Both DCs and OPN appear to be indispensable for granulomatous inflammation; however, there is little information concerning the interaction between OPN and DCs. Monocytes are precursors of myeloid DCs and are recruited to the sites of inflammation where they differentiate into DCs or macrophages. In the present study, we found that OPN is synthesized by monocytes as well as by immature and mature Mo-DCs. Secreted OPN enhances differentiation and maturation of DCs from monocytes to mature DCs with high levels of expression of MHC class II and costimulatory molecules that are necessary for antigen presentation. Furthermore, OPN is important in DC survival. On the basis of these findings, we believe that OPN has an indispensable role in differentiation and survival of DCs. The abnormal cell-mediated immunity and antimicrobial immunity in OPN-deficient mice may be due to impaired function of DCs that failed to mature fully and could not be activated.

The signaling pathways involving OPN are not well understood. However, both RGD-dependent (e.g., αVβ3 integrin) and -independent (e.g., CD44) signaling pathways can serve as receptors for OPN. Although CD44 is a major receptor for hyaluronan (2), it also acts as a receptor for OPN and has multiple bob-RGD binding sites (13). One study showed that anti-CD44 Ab interfered with OPN binding to CD44 on the surfaces of DCs, which partially impaired function and maturation of DCs (11, 32, 35). Lin and colleagues (17) reported that OPN contributes to the survival-promoting activities of cytokines, such as GM-CSF and IL-3, and its signaling pathway occurs through the interaction between CD44 and OPN. We confirmed that anti-CD44 Ab treatment induced apoptosis of Mo-DCs, as observed with anti-OPN Ab-treated DCs (data not shown). For Mo-DCs, the interaction between CD44 and OPN may be important for survival.

It was reported that OPN can inhibit apoptosis of endothelial cells (14), epithelial cells (22), and pro-B cells (28). These reports suggest that OPN acts as a cell survival factor and protects cells from apoptosis. In the present study, we clearly showed that Mo-DCs undergo apoptosis easily in the absence of OPN. Our results are consistent with previous findings for several other cell types (14, 22, 28), suggesting that OPN might be a fundamental factor for cell survival regardless of cell lineage.

DCs undergo apoptosis after finishing antigen presentation, which may be the physiological means of terminating the immune response and preventing prolonged activation of T cells to avoid excessive inflammation (6). Our data suggest that maturation of DCs in response to various factors reduces production of OPN, which may promote apoptosis of DCs. The role of locally synthesized OPN in the survival of DCs appears to be beneficial for maintaining homeostasis at the inflammation site.

In summary, we showed that human Mo-DCs synthesize OPN and that OPN acting in an autocrine and/or paracrine manner contributes to maturation and activation of DCs. Furthermore, OPN promotes survival of DCs as it does for other inflammatory cells. Further characterization of OPN function and the mechanisms of interaction between OPN and inflammatory cells, including DCs, may improve our understanding of inflammatory processes.

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Alteration in Gene Expression Profile by Full-Length Hepatitis B Virus Genome

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Key Words

Hepatitis B virus · Hepatitis B virus-expressing cell · DNA array assay · Corroboration assay · Hepatocarcinogenesis

Abstract

Persistent expression of hepatitis B virus (HBV) proteins is thought to be involved in virus-related hepatocarcinogenesis. Here, we compared the gene expression profile of cells persistently expressing the full-length HBV with that of negative control cells to comprehensively investigate virus-mediated changes in the gene expression of the host cells. RNA samples from both virus-expressing and negative control cells were used for the DNA array assay. DNA array assay and subsequent corroboration assays revealed that expression of 14 of 1,176 genes (1.2%) was altered in response to virus expression. The upregulated genes included CD44, high mobility group protein-I, thymosin beta-10 and 27-kD heat shock protein, while the downregulated genes included NM23-H1, all of which are thought to be associated with the development or progression of carcinoma in the liver or other organs. Furthermore, virus expression resulted in the decrease of two apoptosis-inducing molecules, caspase-3 and BAX, which may also contribute to carcinogenesis through prolonged survival of the host cell. Thus, expression of the virus genome caused carcinogenesis-related changes in host cell gene expression. HBV expression may change the host cell to a malignant phenotype through alterations in the expression levels of a set of genes.

Introduction

Hepatitis B virus (HBV) is a major causative agent of acute and chronic liver diseases. Chronic HBV infection eventually results in more serious liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. HBV is a circular, partially double-stranded DNA virus of approximately 3.2 kb in length that encodes four kinds of viral proteins, i.e. preS/S, precore/core, polymerase and X proteins. Among these HBV proteins, the role of the X protein (HBx) in HBV-mediated pathogenesis has been studied most extensively. HBx displays tumorigenic transforming activity in vitro [3] and in vivo [4]. Also, HBx is thought to considerably modify cellular apoptotic processes under various apoptosis-inducing stimuli [5–7]. HBx acts as a transcription activator for many cellular and virus promoters and enhancers [reviewed in ref. 8]. In

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addition, HBx affects various cellular signal transduction pathways [9–13]. Furthermore, the biologic activities of HBx may be induced by its direct binding to cellular target proteins [14–21].

Another HBV protein, preS/S protein, may also act as a transcriptional transactivator. Both pre-S1 [22] and pre-S2/S [23] proteins activate transcription of a particular gene, suggesting that these proteins, as well as HBx, substantially contribute to modifications of the host cellular function. Therefore, it is important to determine the phenotypic changes in the host cell due to expression of the full-length HBV genome.

Recent advances in DNA array technology make it possible to simultaneously examine the expression levels of hundreds to thousands of genes. In the present study, we compared the gene expression profile of cells persistently expressing full-length HBV (HB611 cells) [24] with that of negative control cells using a DNA array assay, and comprehensively investigated the alterations in the gene expression of host cells in response to the expression of complete HBV proteins.

Materials and Methods

Cell Culture

HB611 cells were established from a human hepatoblastoma cell line, Huh-6, by transfection with the plasmid 3HBneo carrying a 3-tandem repeat of the full length of HBV adr4 strain [24]. HB611 cells are capable of transcribing the pregenome RNA and other viral mRNAs from the integrated HBV DNA through regulation by their own promoter/enhancer, followed by constitutive production of viral proteins and the release of Dane-like particles into the culture medium [24]. Huh-6 neo cells were generated by transfection with only the neomycin-resistant gene and used as a negative control. Both HB611 and Huh-6 neo cells were kindly provided by Prof. K. Matsubara (Institute for Molecular and Cellular Biology, Osaka University Graduate School of Medicine, Osaka, Japan). These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B and 200 μg/ml G418 sulfate (Gibco BRL/Life Technologies, Inc., Tokyo, Japan) at 37° under an atmosphere of 5% CO_2-95% air.

DNA Array Analysis

In the present study, a commercially available DNA array system (Atlas Human Array 1.2, Clontech Laboratories, Inc., Palo Alto, Calif., USA) was used for the analysis. This is a broad-coverage DNA array system that is capable of simultaneously analyzing the 1,176 genes examined in this study. The DNA array procedures were conducted according to the manufacturer's instructions. Briefly, total RNA was extracted from both HB611 and Huh-6 neo cells in a confluent state with TRIZOL reagent (Gibco BRL/Life Technologies), and the mRNA was selected using an oligo-dT column (Roche Diagnostic Co. Ltd., Tokyo, Japan). The mRNA sample was treated with RNase-free DNase I (Promega Co., Madison, Wisc., USA), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitation with ethanol. The resulting mRNA sample (1 μg) was used for the DNA array analysis. After cDNA synthesis, parallel hybridization with cDNA samples derived from HB611 and Huh-6 neo cells was performed using two identical membranes loaded with the gene probes. Finally, the arrays were exposed for 24 h using the bioimaging analyzer BAS-2500 (Fuji Photo Film Co. Ltd., Tokyo, Japan). Quantitation of the signal intensity of each gene was performed using Atlas Image software (Clontech Laboratories).

Reverse Transcription-Polymerase Chain Reaction Analysis

For the reverse transcription (RT)-polymerase chain reaction (PCR) assay, total RNA was extracted from both the HB611 and Huh-6 neo cells with TRIZOL reagent (Gibco BRL/Life Technologies), as described above. After RNase-free DNase I (Promega) treatment, cDNA was synthesized using mutated Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, Toyobo, Co. Ltd., Osaka, Japan) and oligo $(dT)_{20}$ primer (Toyobo). Table 1 shows the primers for the PCR analysis used in this study. An aliquot of the cDNA product was subjected to PCR reaction (94° for 30 s, 55° for 1 min and 72° for 2 min), followed by a final extension at 72° for 10 min. PCR reaction for 34, 31, 28 and 25 cycles was performed in each experiment, and the appropriate cycles for the comparison of the gene expression level between HB611 and Huh-6 neo cells were determined for each gene. As an internal control, β-actin mRNA was also examined. The PCR product was separated using agarose gel electrophoresis and visualized with ethidium bromide staining under an ultraviolet lamp.

Western Blot Analysis

For the Western blot analysis, cells in a confluent state were lysed and separated using SDS-PAGE. After transfer onto a nitrocellulose membrane (Hybond-P, Amersham Pharmacia Biotech Co. Ltd., Buckinghamshire, UK), the membrane was blocked with 5% milk. The membrane was then incubated with the primary antibody, followed by further incubation with immunoglobulin coupled with horseradish peroxidase as a secondary antibody. Finally, the proteins were detected by chemiluminescence (Supersignal, Pierce Chemical, Rockford, Ill., USA). The following antibodies were used in this study: anti-27-kD heat shock protein (HSP-27; Upstate Biotechnology, Lake Placid, N.Y., USA), anti-NM23-H1 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA), anti-caspase-3 (MBL Co. Ltd., Nagoya, Japan) and anti-BAX (Santa Cruz Biotechnology).

Results

Results of the DNA Array Analysis

To investigate the changes in the gene expression profile caused by transfection of the full-length HBV DNA, DNA array analysis was performed using mRNA samples derived from HB611 and Huh-6 neo cells. Genes with a HB611 to Huh-6 neo signal intensity ratio of 3 or greater were regarded as upregulated, whereas genes with an HB611 to Huh-6 neo signal intensity ratio of 0.33 or less were regarded as downregulated. Figure 1 shows the com-

Table 1. Sequences of primers for PCR amplification used in this study

Genes		Primer sequence (5′ to 3′)
CD44	sense	CATCTACCCCAGCAACCCTA
	antisense	CTTCTGCCCACACCTTCTTC
HMG-I	sense	AGTGAGTCGAGCTCGAAGTC
	antisense	GTCTCTTAGGTGTTGGCACT
TB-10	sense	GGAAATCGCCAGCTTCGATA
	antisense	AATCCCTCCAGGATCTTAGG
α_1-AT	sense	GGGTCAACTGGGCATCACTA
	antisense	CCATGAAGAGGGGAGACTTG
α_1-AG	sense	AGAGTACCAGACCCGACAGG
	antisense	CTCTCCTTCTCGTGCTGCTT
IGFBP-1	sense	GAGAGCACGGAGATAACTGAGG
	antisense	AACCACTGTACCTCTCGGAAGC
BTEB2	sense	ACTTACTTTCCCCCGTCACC
	antisense	CAGCCTTCCCAGGTACACTT
TOPO-IIα	sense	TGTCACCATTGCAGCCTGTA
	antisense	GTCGAGAAGGGTATAATAGG
HIF-1α	sense	TGTAATGCTCCCCTCACCCAACGAA
	antisense	GTGACCCTGATAATCCGAGTCCACT
ROR1	sense	CCTCATGACAGAGTGCTGGA
	antisense	GAGGACCTGTTGGCTGGTAG
β-Actin	sense	ACACTGTGCCCATCTACGAGG
	antisense	AGGGGCCGGACTCGTCATACT

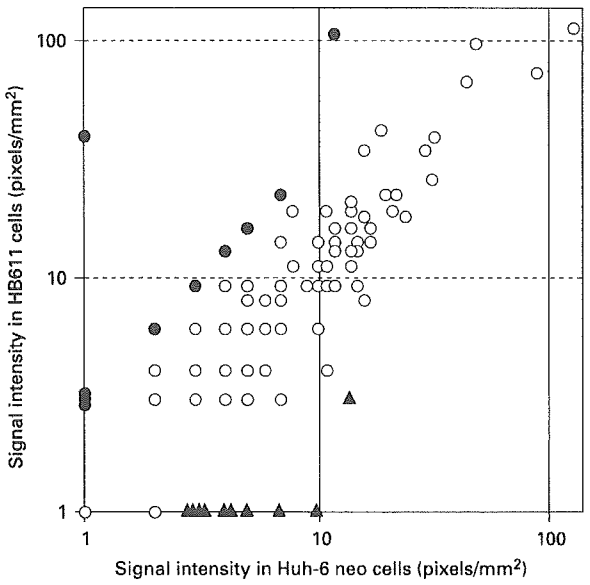

Fig. 1. Comparison of gene expression levels between Huh-6 neo and HBV-expressing HB611 cells in DNA array analysis. The signal intensities of each gene were quantitated with Atlas Image software (Clontech Laboratories). ● = Genes upregulated by HBV expression, with a HB611 to Huh-6 neo signal intensity ratio ≥ 3; ▲ = genes downregulated by HBV expression, with a HB611 to Huh-6 neo signal intensity ratio ≤ 0.33; ○ = genes whose expression levels were not altered by HBV expression.

parison of the gene expression levels between HB611 and Huh-6 neo cells. The expression of 10 genes was significantly enhanced, whereas the expression of 10 genes was reduced due to persistent expression of HBV. Thus, 20 of the 1,176 genes (1.7%) examined in this study were initially judged to be altered by HBV expression in the DNA array analysis.

Results of Corroboration Assays by RT-PCR and Western Blot

For the 20 genes initially regarded to be HBV responsive in the DNA array analysis, RT-PCR analysis (for 14 genes) or Western blot analysis (for 6 genes) was further performed for corroboration. The RT-PCR results are shown in figure 2. In the RT-PCR assay, 7 genes, i.e. CD44, high mobility group protein-I (HMG-I), thymosin beta-10 (TB-10), alpha-1-antitrypsin (α_1-AT), alpha-1-acid glycoprotein 1 (α_1-AG), insulin-like growth factor-binding protein 1 (IGFBP-1) and basic transcription element-binding protein 2 (BTEB2), were upregulated, whereas 3 genes, i.e. DNA topoisomerase II alpha (TOPO-IIα), hypoxia-inducible factor 1 alpha (HIF-1α) and protein-tyrosine kinase transmembrane receptor ROR1, were downregulated due to expression of HBV proteins. In addition, Western blot analysis revealed that HBV expression induced 1 upregulated gene, HSP-27, and 3 downregulated genes, NM23-H1, caspase-3 and BAX (fig. 3). Expression of the remaining 6 genes was not different between HB611 and Huh-6 cells by RT-PCR assay (4 genes) or by Western blot (2 genes), and these genes were regarded as 'false positives' of the DNA array analysis. The specificity of our DNA array analysis was 70% (14 of 20 genes). The high specificity indicates that the DNA array analysis used in this study was a reliable experimental method to simultaneously examine the expression levels of many genes. The 14 HBV-responsive genes identified in this study are summarized in table 2.

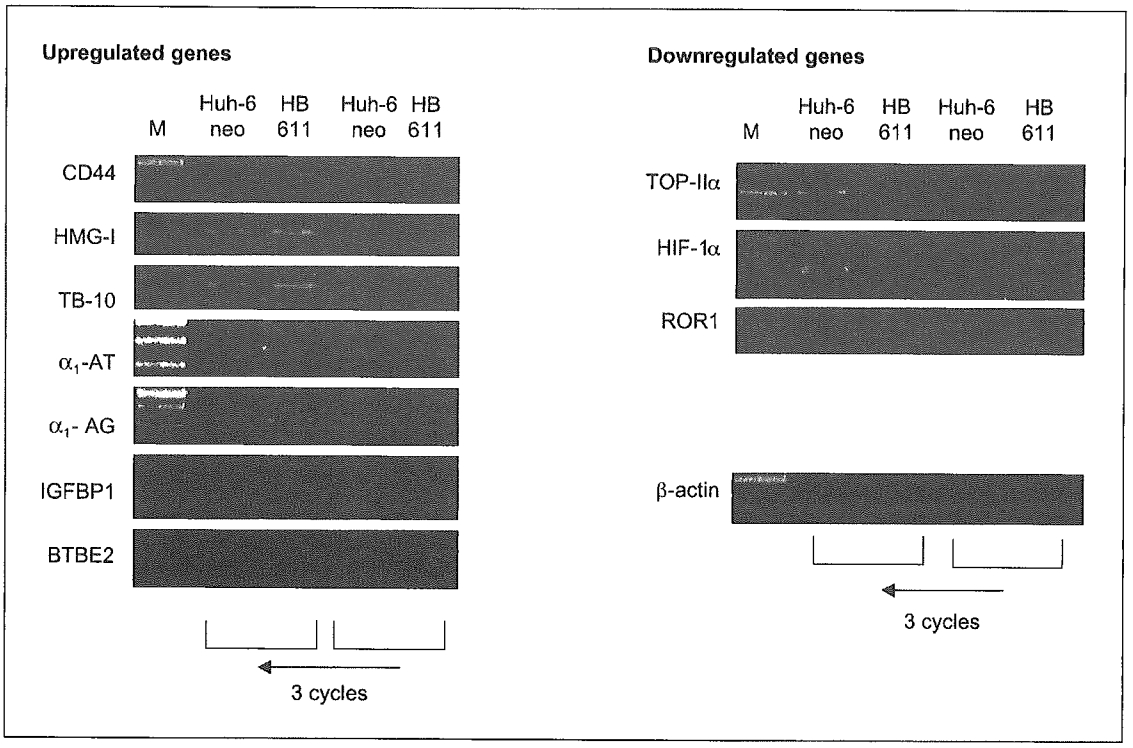

Fig. 2. RT-PCR assay for the detection of HBV-responsive genes. Total RNA samples were extracted from Huh-6 neo and HB611 cells and used for RT-PCR assay. The left panel represents upregulated genes (8 genes), whereas the right panel represents downregulated genes (6 genes). The right bottom panel shows the β-actin mRNA as an internal control. M = 100-bp ladder.

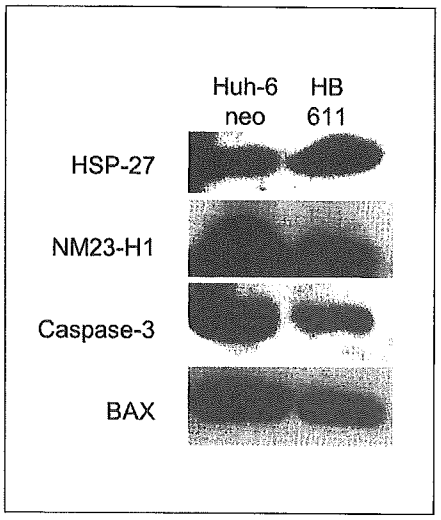

Fig. 3. Western blot analysis for the detection of HBV-responsive genes. Total cellular lysates were extracted from Huh-6 neo and HB611 cells and subjected to Western blot analysis to examine HSP-27, NM23-H1, caspase-3 and BAX expression levels.

Discussion

In the present study, we identified 14 genes whose expression levels were altered by expression of the full-length HBV DNA through the screening of 1,176 genes using the DNA array method. Among these HBV-responsive genes, there were 6 genes, CD44, NM23-H1, BAX, HMG-I, TB-10 and HSP-27, which have been suggested to be closely associated with the development or progression of HCC or other kinds of carcinomas. CD44 is a cell surface glycoprotein that possesses functions in cell-cell and cell-matrix adhesions. High levels of CD44 expression are related to the invasive and metastatic potential of HCC [25, 26]. HBx induces metastatic potential by modifying CD44-dependent migratory behavior, as determined using HBx-overexpressing cultured cells [27]. NM23-H1, a nucleotide diphosphate kinase, is an antimetastatic molecule [28]. In HCC, the reduced expression of NM23-H1 is closely correlated with the presence of intrahepatic metastasis [29] and higher recurrence rates after surgical resection [30]. HMG-I is involved in the regula-

tion of chromatin structure and function, and regulates transcription activity by interacting with several transcription factors. Elevated expression of HMG-I is frequently observed in various carcinomas, such as thyroid tumors [31], colorectal carcinomas [32] and pancreatic duct cell carcinomas [33]. In addition, the forced expression of the HMG-I gene in cultured epithelial cells leads to a malignant and metastatic phenotype of the cells [34]. TB-10 is an acidic polypeptide originally isolated from the calf thymus, and belongs to the β class of the thymosin family. Overexpression of TB-10 is frequently detected in colon, breast, ovarian and uterine carcinomas and germ cell tumors [35]. HSP-27, a member of the heat shock protein family, is thought to have an important role in the regulation of intracellular homeostasis. HSP-27 expression might be associated with a poor clinical outcome in prostate [36] and breast cancers [37], suggesting that overexpression of HSP-27 is linked to the aggressiveness of malignant tumor cells. Transient transfection of HBx into cultured cells enhances expression of HSP-27 [38].

The levels of two acute-phase response proteins, α_1-AT and α_1-AG, were also increased by HBV expression in the present study. Serum α_1-AT and α_1-AG levels tend to be higher in patients with HCC than in patients with chronic hepatitis without HCC [39]. Furthermore, a high level of serum α_1-AT might also be significantly correlated with shorter survival [40].

Thus, a series of previous studies in either a clinical setting or laboratory investigations suggest that enhanced expression of CD44, HMG-I, TB-10, HSP-27, α_1-AT and α_1-AG, and reduced expression of NM23-H1 might be involved in the acceleration of carcinogenesis in the liver or other organs. These findings suggest that each of these alterations in gene expression levels have an important role in the course of carcinogenesis, although the precise mechanisms through which cells would gain a malignant phenotype have not been fully clarified. It is noteworthy that such carcinogenesis-related changes in gene expression levels are caused by expression of the full-length HBV genome, suggesting that persistent expression of HBV might accelerate hepatocarcinogenesis accompanied by alterations in gene expression levels of the host liver cell.

Furthermore, HBV expression suppressed the expression levels of two important apoptosis-inducing molecules, caspase-3 and BAX. Caspase-3, a member of the cysteine protease family, activates its target molecules by proteolytic cleavage and has a crucial role in cellular apoptosis. Transfection of the HBx gene into cultured cells inhibits caspase-3 activity and results in the resistance of cellular apoptosis under various stimuli [7]. Inthe present study, we demonstrated that HBV expression reduced caspase-3 transcription and expression levels. BAX is a proapoptotic member of the BCL-2 family. Downregulation of BAX is observed in HCC tissues with overexpression of the tumor suppressor p53 [41]. In light of this, the HBV-mediated suppression of caspase-3 and BAX might result in prolonged survival of the host cell and contribute to carcinogenesis in the liver.

In the present study, 5 additional HBV-responsive genes were identified. IGFBP-1 takes part in the regulation of the function of insulin-like growth factor (IGF) by binding to IGF [42]. The modulatory effect of IGFBP-1 on the mitogenic activity of IGF, however, has not been fully clarified in the liver cell. TOPO-IIα is a nuclear enzyme that changes the topology of DNA and is essential for chromosome segregation at mitosis. High expression levels of TOPO-IIα are reported in lung cancer [43], in contrast to the suppression of TOPO-IIα by HBV expression observed in the present study. HIF-1α is involved in the transcriptional regulation of a variety of genes related to angiogenesis [44]. HBV expression, however, sup-

Table 2. HBV-responsive genes determined by DNA array screening and the corroboration assays

Genes upregulated by HBV expression (n = 8) (HB611 to Huh-6 neo ratio ≥ 3)
CD44 antigen (M59040)
High mobility group protein-I (M23619)
Thymosin beta-10 (M92381)
27-kD heat shock protein (X54079)
Alpha-1-antitrypsin precursor (X02920)
Alpha-1-acid glycoprotein 1 precursor (X02544)
Insulin-like growth factor-binding protein 1 (M31145)
Basic transcription element-binding protein 2 (D14520)
Genes downregulated by HBV expression (n = 6) (HB611 to Huh-6 neo ratio ≤ 1/3)
Metastasis inhibition factor NM23 (X17620)
Caspase-3 (U13737)
Apoptosis regulator BAX (L22474)
DNA topoisomerase II alpha (J04088)
Hypoxia-inducible factor 1 alpha (U22431)
Protein-tyrosine kinase transmembrane receptor ROR1 (M97675)

Another 6 genes were initially judged to be HBV-responsive, but were subsequently found to be false positive by RT-PCR or Western blot analysis for corroboration. Numbers in parentheses represent the GenBank accession numbers.

presses HIF-1α levels, despite the fact that angiogenesis has a key role in the progression of malignant cells. As for ROR1, an orphan cell surface receptor with strong homology to the tyrosine kinase domain of growth factor receptors [45], and BTEB2, a transcription factor involved in phenotypic changes of smooth muscle cells [46], their functions in the liver have not yet been clarified. Thus, the biologic significance of HBV-mediated alterations in these 5 genes remains unclear, especially with respect to carcinogenesis.

In conclusion, our DNA array results suggest that HBV expression induces the host cell to adopt a malignant phenotype through alterations in the expression levels of a set of carcinogenesis-related genes. These findings may serve as a first step toward the comprehensive understanding of HBV-mediated development or progression of HCC. It remains unclear, however, which parts of the HBV region are responsible for the change in the expression level of each HBV-responsive gene. Also, the detailed functions of these HBV-responsive genes in the acceleration of carcinogenesis should be clarified.

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6 Apoptosis resistance in the tumor cells of the liver and gastrointestinal tract

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Abstract

Cell death is executed along several pathways. Different from another form of cell death called necrosis, a morphological-defined cell death, called apoptosis, has been recently observed. Apoptosis or programmed cell death is multi-step, multi-pathway cell death program. It can be triggered by several stimuli, such as intracellular stress and receptor-mediated signaling, and is tightly controlled by various proteins. Apoptosis plays an important role in physiological processes, especially for the development and for the immune system. Moreover, many diseases are associated with too much or too little apoptosis. In this regard, cancer development is attributed to the latter. Apoptosis resistance of tumor cells leads to escape from immune surveillance and influences on immuno-therapy. Furthermore, apoptosis resistance may cause resistance to chemotherapy and γ-irradiation. Since a variety of

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mechanisms, underlying apoptosis resistance have been reported, combination of therapeutic strategies is required to circumvent apoptosis resistance of tumor cells.

Introduction

Cells have an intrinsic mechanism of self-destruction, called apoptosis or programmed cell death, which is a fundamental biological process with tremendous implications for the maintenance of tissue homeostasis. It plays a critical role in many physiological processes, especially for the development and for the immune system (1).

On the molecular levels, apoptosis program can be divided into three parts: initiation, execution, and termination. Apoptosis is initiated by death ligands, or by a variety of stimuli including chemotherapy, and γ-irradiation. Execution phase is characterized by membrane inversion, fragmentation of DNA, chromatin condensation. In the termination phase, apoptotic bodies are engulfed by phagocytes (2). Deregulation of apoptosis can disrupt the normal balance of cell proliferation and cell death, resulting in a pathological state. In this context, defects in the apoptosis-inducing pathways eventually lead to expansion of the population of neoplastic cells. Apoptosis resistance may cause escape of tumor cells from immune surveillance. Since chemotherapy and γ-irradiation exert their actions primarily by inducing apoptosis, defects in the apoptosis signaling make the tumor cells to be resistant to these therapies. In this way, apoptosis resistance is clinically significant. To comprehend the mechanisms of apoptosis resistance would provide a deeper insight into carcinogenesis and lead to the development of a new therapeutic strategy based on modulation of apoptosis sensitivity.

I. Mechanisms of apoptosis

a) Initial step of apoptosis

Caspases are a growing family of aspartyl-specific cysteine protease, synthesized as zymogens. So far, 14 mammalian caspases have been identified (3). Upstream signal converts these precursors into mature caspases. In turn, they cleave their substrates, leading to the biochemical and morphological changes of apoptosis. Thus, caspases are crucial for execution of apoptosis. Three alternative pathways of apoptosis have been revealed according to the initiator caspases (4); the first pathway mediated by death receptor (extrinsic pathway) involves caspase 8 and 10, the second one mediated by mitochondria (intrinsic pathway or mitochondrial pathway) involves caspase 9, and the last one mediated by endoplasmic reticulum involves caspase 12(5)(Figure 1).

The death receptors are members of TNF/NGF-receptor superfamily including Fas (CD95), TNF-αR, TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1), and TRAIL-R2. They are activated through their natural ligands which have co-evolved as death ligand family corresponding to the death receptors. The death receptor has an intracellular death domain (DD), which is essential for transduction of apoptotic signal (Figure 2). On the contrary, decoy receptors including TRAIL-R3 and TRAIL-R4, are closely related to the death receptors, but are lack of a functional death domain (6). When the death ligands bind themselves to their respective receptors, the death domains attract the intracellular adaptor protein FADD (Fas-associated death domain), which in turn recruits the inactive proform members of the caspase family (pro-caspase 8, pro-

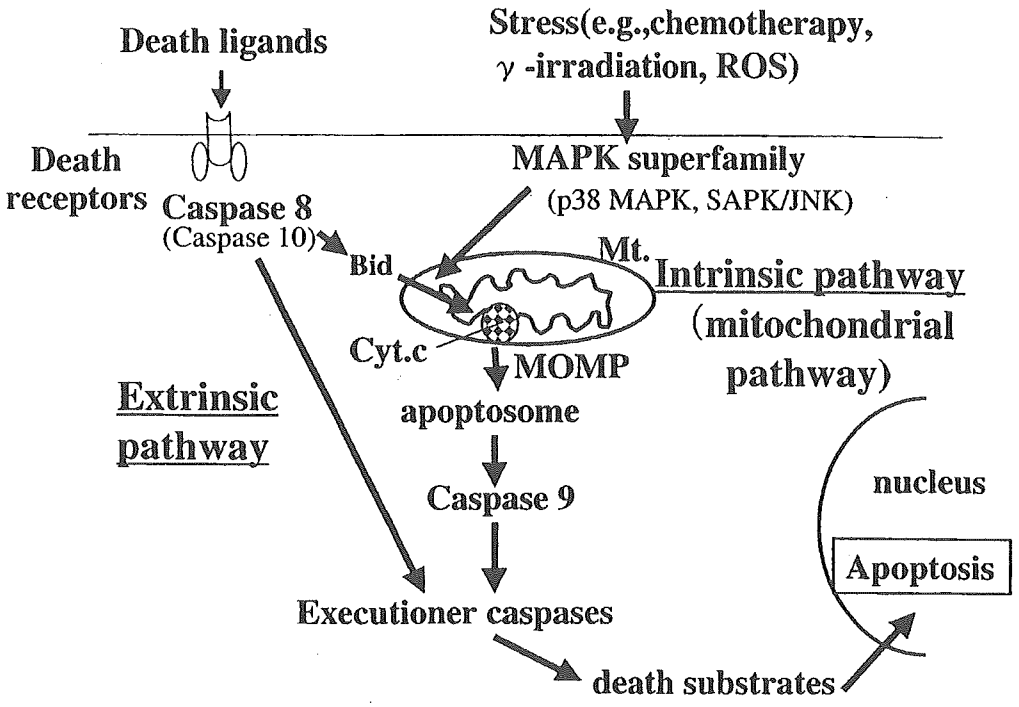

Figure 1. The two major apoptotic signaling pathways. Apoptosis can be initiated by two major pathways; the first pathway is mediated by death receptors (extrinsic pathway), and the second one is mediated by mitochondria (mitochondrial or intrinsic pathway). In these pathways, initiator caspases (caspases 8, 10 and caspase 9 in extrinsic pathway, mitochondrial pathway, respectively) are activated by apoptotic stimuli, followed by activation of executioner caspases. Finally, activated executioner caspases cleave their substrates, leading to apoptosis. It has been shown that caspase 8 cleaves the Bcl-2 family, Bid, accompanied by induction of mitochondrial pathway. Such cross-talk of two apoptotic pathways is observed in type II cells. ROS, reactive oxgen species; Mt., mitochondria; Cyt.c, cytochrome c; MOMP, mitochondrial outer membrane permeabilization.

caspase10 in this case) to form the DISC (death-inducing signaling complex) (Figure 2). Finally, pro-caspase 8 and pro-caspase10 are cleaved and yield active initiator caspases. Recently, cells have been classified into two types, according to the different mechanisms whereby signaling from death receptors is transduced: type I and type II. In type I cells, the amount of active caspase 8 formed at DISC is high enough to initiate the executioner apoptotic caspase. In type II cells, however, the amount is so little that mitochondria play as amplifiers to initiate apoptosis (7). In this context, BID, which is a pro-apoptotic member of the Bcl-2 family, is cleaved by active caspase 8, and translocates to mitochondria in order to amplify the apoptotic signal. The examples of such type II cells are: hepatocytes and liver cancer cells.

On the other hand, in the mitochondrial pathway, caspase activation is closely linked to MOMP (mitochondrial outer membrane permeabilization). A variety of pro-apoptotic signaling molecules and various pathological stimuli, such as chemotherapy, γ-irradiation, ROS (reactive oxygen species), converge on mitochondria to induce MOMP. In turn, MOMP results in the release of cytochrome c to the cytosol. Cytochrome c forms a complex, termed as apoptosome, with Apaf-1 (apoptotic protease activating factor-1), ATP and inactive initiator pro-caspase 9. Within this complex, pro-caspase 9 is activated to be caspase 9(8)(Figure 1).

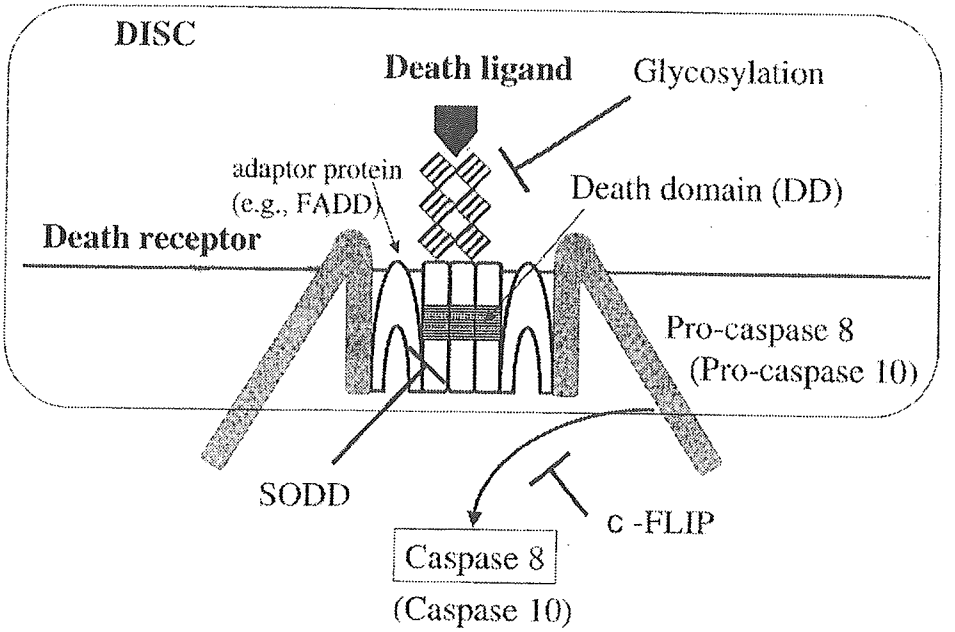

Figure 2. Regulation of death receptor signaling (extrinsic pathway). At death receptor level, the glycosylation status of death receptor modulates death receptor-mediated apoptotic signaling. Moreover, FLIP and SODD interact with pro-caspase8, DD of death receptor, respectively, resulting in inhibition of apoptotic signaling. For details, refer to the text. FADD, Fas-associated death domain; SODD, silencer of death domain; FLIP, FADD-like interleukin-1 β-converting enzyme-like protease inhibitory proteins; c-FLIP, cellular FLIP; DISC, death-inducing signaling complex.

b) Execution of apoptosis

Once initiator caspases are activated, they cleave and activate executioner caspases, mainly caspase 3, caspase 6 and caspase 7. The active executioner caspases then cleave each other. Eventually, such caspases start to cleave cellular substrates - the death substrates -, and which distinctively leads to biochemical and morphological changes. For instance, cleavage of the ICAD (inhibitor of caspase-activated deoxyribonuclease) causes the release of the endonuclease, which travels to the nucleus to fragment DNA (9).

In contrast to these proteotypic caspase-dependent apoptosis pathways, other cell death pathways that do not require caspase activation have been recently identified. Moreover, autophagic execution pathways of cell death may be engaged without either the involvement of caspases or morphological changes of apoptosis (10).

c) Regulation of apoptosis

Apoptosis is strictly controlled by a variety of mechanisms at different levels.
At death receptor level, the glycosylation status of Fas (CD95) modulates Fas-mediated apoptosis (11). In addition, FLIPs (FADD-like interleukin-1 β-converting enzyme-like protease inhibitory proteins) interfere with the initiator caspases; two splice variants - a long form ($FLIP_L$) and a short form ($FLIP_S$) - have been identified in human cells (12). The long form ($FLIP_L$) shares structural homology with pro-caspase 8, which allows these forms to bind to the DISC, and therefore, inhibits the activation of pro-caspase 8(Figure 2).