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Emergence of nuclear heparanase induces differentiation of human mammary cancer cells ☆

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Abstract

The study of epithelial differentiation touches upon many modern aspects of biology. The epithelium is in constant dialogue with the underlying mesenchyme to control stem cell activity, proliferation in transit-amplifying compartments, lineage commitment, terminal differentiation and, ultimately, cell death. There are spatially distinct compartments dedicated to each of these events. Recently we reported that heparanase is expressed in nucleus as well as in the cytoplasm and that nuclear heparanase seems to be related to cell differentiation. In this study, we investigated the role of nuclear heparanase in differentiation by transducing human mammary epithelial cancer cells with heparanase which was delivered specifically into nucleus. We observed that expression of nuclear heparanase allowed the cells to differentiate with the appearance of lipid droplets. This finding supports the idea that heparanase plays a novel role in epithelial cell differentiation apart from its known enzymatic function.

Keywords: Heparanase; Nucleus; Differentiation; Epithelial cancer; MCF-7

Heparanase is an endo-β-glucuronidase that specifically cleaves carbohydrate chains of heparan sulfate proteoglycans (HSPG), which are important components of extracellular matrix (ECM) [1–5]. Most previous studies reported that heparanase is involved in invasion and metastasis of many types of cancers by mediating the degradation of HSPG in the basement membrane (BM) and ECM [1–3,6,7]. In addition, heparanase expression has been associated with an aggressive malignant phenotype and adverse prognosis in cancer patients. Increased heparanase expression was demonstrated in several human primary cancers, including colon [8], stomach [9,17], esophagus [10], bladder [11], pancreas [12], liver [13], prostate [14], malignant melanoma [15], and breast [16].

On the other hand, recent studies claimed that heparanase is also expressed in the nucleus [17–19] and that nuclear heparanase is statistically correlated with the expression of a differentiation marker in the esophagus [19].

☆ *Abbreviations:* BM, basement membrane; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycans.

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Induction of terminal differentiation represents a promising alternative to conventional chemotherapy for certain malignancies. For example, the retinoic acid receptor α, which plays an important role in the differentiation and malignant transformation of cells of the myelocytic lineage, has been used as a target for intervention in acute promyelocytic leukemia [20,21]. Differentiation therapy with all-*trans* retinoic acid has become the standard of care for this disease.

Thus, induction of cell differentiation is potentially very promising for cancer therapeutics in other types of cancers as well. Here we report that heparanase emergence in the nucleus regulates cell differentiation in a human breast cancer cell line.

Materials and methods

Cell culture. The human breast cancer cell line MCF-7 was obtained from Dainippon Pharmaceutical MCF-7 cells were in maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin G, and 100 μg/ml streptomycin, sodium pyruvate, and non-essential amino acids for Eagle's MEM in a humidified 5% CO_2/air atmosphere at 37 °C.

Plasmid constructs: pShooter/nuc-hpa vectors. pShooter Vector (pCMV/myc^C vectors) was purchased (Invitrogen Japan, Tokyo). To target heparanase specifically into the nucleus, we constructed heparanase pShooter cytoplasmic forms. pCMV/myc/Nuc vector contained three copies of the nuclear localization signal of SV40 large T antigen. All heparanase coding sequences were amplified by PCR with sense primer Hep. *Nco*I.S (5′-CACCATGGCCATGCTGCTGCGCTCGAAG-3′) and anti-sense primer Hep. *Xho*I.AS (5′-ATCTCGAGGATGCAAGC AGCAACTTTGG-3′), which included adaptors for *Nco*I (sense) and *Xho*I (antisense) restriction endonuclease sites. The heparanase insert was cloned into the *Nco*I–*Xho*I sites of the pCMV/myc/Nuc vector. One clone for the nuclear pCMV/myc/Heparanase was selected and designated pShooter/nuc-hpa.

Electroporation. Fifty micrograms of pShooter/nuc-hpa plasmid was mixed with 8×10^6 cells for 5 min and then electroporated at 250 V and 1000 μF with Gene Pulser II (Bio-Rad). Cells were immediately transferred to complete medium pre-warmed to 37 °C and plated into chamber slides. After 24 h, the medium was changed.

Double staining: immunohistochemistry and Oil Red O staining. Cells grown on chamber slides for 2 days after transfection were fixed with paraformaldehyde, washed three times with PBS, treated with rabbit serum for 10 min at room temperature, and incubated overnight at 4 °C with anti-human heparanase mouse monoclonal antibody raised against recombinant human heparanase. Identification of the distribution of the primary antibody was achieved by subsequent application of biotinylated anti-primary antibody and streptavidin peroxidase. Immunostaining was developed using DAB/hydrogen peroxidase solution (Histofine DAB substrate kit; Dako Japan, Tokyo, Japan). As soon as cells were washed with distilled water, they were stained with Oil Red O (Sigma Chemical) for 10 min according to published procedures [22,23,30], and counterstained with Mayer's hematoxylin solution (Sigma Chemical). As a negative control, some cells were subjected to normal serum blocking and omission of the primary antibody. Immunohistochemistry was conducted using the Histofine SAB PO kit (Nichirei, Tokyo) and the instructions provided by the manufacturer.

Evaluation of differentiation. The cells were fixed with paraformaldehyde 2 days after the transfection, washed three times with PBS, and stained with Oil Red O (Sigma Chemical) for 10 min according to published procedures [22,23,30]. Cells were then counterstained with Mayer's hematoxylin solution (Sigma Chemical), and each coverslip was mounted onto a glass side. Specimens were examined and photographed. By counting the number of cells containing 10 or more Oil Red O-stained lipid droplets, the percentage of cells staining positive for lipid droplet accumulation after treatment was determined.

Confocal microscopy. To determine the localization of heparanase protein in MCF-7 cells, the cells grown on chamber slides were fixed with paraformaldehyde and then washed three times with PBS. Endogenous peroxidase was blocked by incubating the cells in 3.0% H_2O_2 in methanol for 10 min and washed three times with PBS. After blocking of non-specific reactivity with rabbit serum for 10 min at room temperature, sections were incubated overnight at 4 °C with the anti-human heparanase mouse monoclonal antibody raised against recombinant human heparanase. Cells were washed three times with PBS and then incubated with FITC-conjugated anti-mouse IgG1 rat monoclonal antibody for 30 min at 4 °C. Cells were washed three times with PBS and analyzed by a Carl Zeiss LSM 510.

Electron microscopy. Cells grown in a flask were harvested, postfixed in 2.5% glutaraldehyde, and postfixed further in a solution of 1% OsO_4. These were dehydrated and embedded according to standard procedures, and thin sections were cut stained with uranyl acetate–lead citrate, and examined with EF LEO912 transmission electron microscope.

Statistical analysis. Data in figures means ± SD of at least three different experiments performed in triplicate. Evaluation of differentiation was compared by Student's t test, and $p < 0.05$ was considered significant.

Results

Nuclear heparanase is expressed by transfection with pShooter/nuc-hpa vectors in MCF-7 cells

Fig. 1 shows our fundamental strategy to transduce heparanase in nucleus using pShooter/nuc-hpa vector system. Based upon the transfection assay, we first measured heparanase overexpression in MCF-7 cells. In wild type MCF-7 cells, heparanase was localized only in cytoplasm (Fig. 2A) but not in nucleus. Significant expression of nuclear heparanase was detected in the cells that were transfected with pShooter/nuc-hpa vectors (Figs. 2B and C).

Emergence of nucleic heparanase induces the expression of lipid droplets in MCF-7

Next, we assessed the role of nuclear heparanase in cancer cells. MCF-7 cells were transfected with pShooter/nuc-hpa vectors and then analyzed by double staining of heparanase and lipid droplets. Half of pShooter/nuc-hpa vector-transfected cells showed clearly strong heparanase expression in nucleus and the others scarcely presented nuclear heparanase expression because of the transfection efficiency in our hands. Lipid droplets were detected in the cytoplasm roughly in accord with nuclear heparanase expression (Fig. 2C). In contrast, MCF-7 cells, which did not express heparanase in nucleus, hardly showed lipid droplets (Fig. 2D).

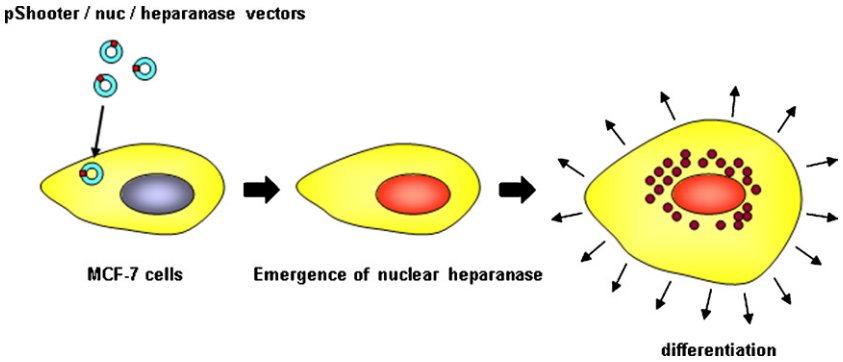

Fig. 1. Schematic procedure for transduction of heparanase into nucleus. Heparanase can be expressed in the nucleus of MCF-7 cells after the transfection with pShooter/nuc-hpa vectors. Transfection efficiency by electroporation was approximately 50% in average.

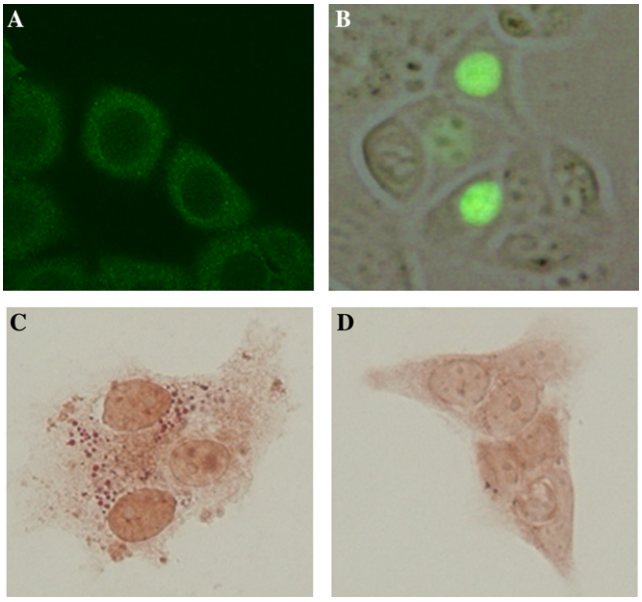

Fig. 2. Emergence of nuclear heparanase and differentiation in MCF-7 cells. (A) Immunofluorescent staining of heparanase (confocal microscopy) in the wild type MCF-7. Heparanase is localized only in cytoplasm and is not expressed in nucleus. (B) Immunofluorescent staining of heparanase (confocal microscopy) in pShooter/nuc-hpa transfected MCF-7 cells. Heparanase is expressed in nucleus. (C,D) Double staining of heparanase (immunohistochemistry) and lipid droplets (Oil Red O staining) in MCF-7 cells. (C) pShooter/nuc-hpa vectors had been transfected to the cells and many of them expressed heparanase in both of nucleus and cytoplasm but the nucleic heparanase expression is much more enhanced than the cytoplasm, and lipid droplets are greatly increased as a series of granules in cytoplasm in accord with nucleic heparanase expression. (D) pShooter/nuc-hpa vectors had not been transfected to the cells, and both specific heparanase expression in nucleus and lipid droplets in cytoplasm was not seen.

In electron microscopy, lipid droplets were very clearly detected in pShooter/nuc-hpa vector-transfected cells (Fig. 3B), but few were detected in control cells (Fig. 3A). Furthermore, the morphology of pShooter/nuc-hpa transfected cells looked spread and flattened (Fig. 3B), so the volume of cytoplasm in the cells was larger than in control or untransfected cells.

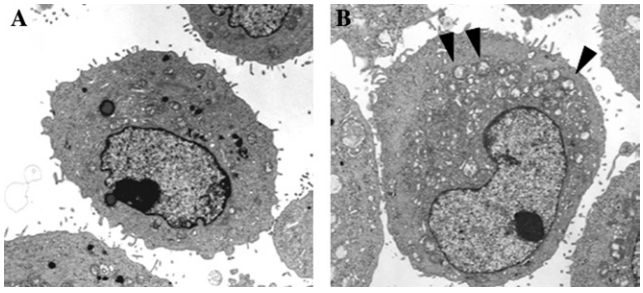

Fig. 3. Ultrastructure of MCF-7 cells in electron microscopy. (A) The control vector-transfected MCF-7 cells which expressed no heparanase in nucleus did not express newly lipid droplets. (B) The MCF-7 cells transfected with pShooter/nuc-hpa vectors, which expressed heparanase in nucleus, presented a lot of lipid droplets both of large and small size in their cytoplasm (arrowheads).

Evaluation of differentiation showed that nuclear localization of heparanase is a significant factor for the differentiation in MCF-7cells

Finally, we made an assessment of differentiation in the cells by counting the cells which expressed lipid droplets in their cytoplasm as described under Materials and methods. Compared to control vector-transfected cells (Fig. 4A), the pShooter/nuc-hpa vector-transfected cells (Fig. 4B) displayed an approximately 4-fold increase in the number of lipid-positive cells and also an increase in the number of lipid droplets per cell (Fig. 4C). In addition, we observed that the pShooter/nuc-hpa vector-transfected cells tended to grow slower than control cells (data not shown). This finding suggests that heparanase appearance in nucleus may be important to induce differentiation of MCF-7 cells.

Discussion

Our present study showed that overexpression of heparanase in nucleus extensively correlates with differentiation of MCF-7 cells as measured by Oil-Red O staining. Previous studies have demonstrated that hepa-

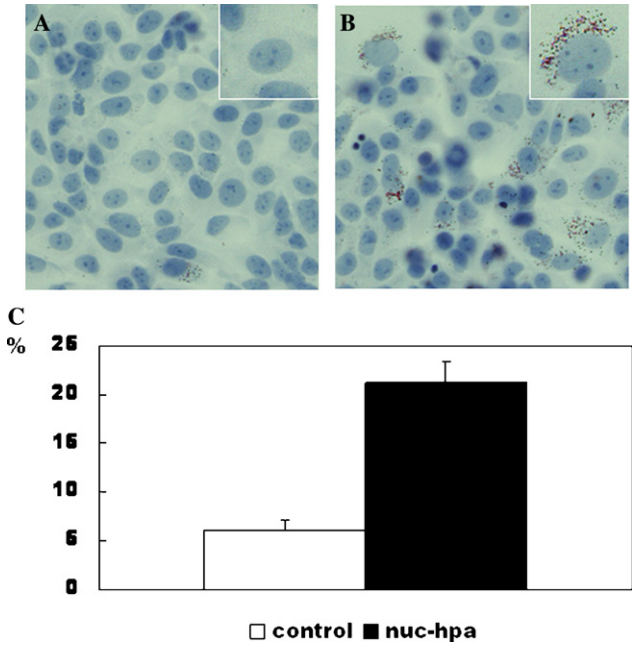

Fig. 4. Evaluation of the differentiation in the cells which express nucleic heparanase. (A) The control vector-transfected MCF-7 cells hardly presented lipid droplets in their cytoplasm. (B) The MCF-7 cells which had been transfected with pShooter/nuc-hpa vectors expressed increased accumulation of lipid droplets in their cytoplasm. (C) Evaluation of differentiation. Compared with the number of cells which had ten or more Oil Red O-stained lipid droplets in their cytoplasm between the control MCF-7 cells and the cells that are transfected with pShooter/nuc-hpa vectors, significantly larger number of cells in the latter group expressed enough lipid droplets in cytoplasm than the control ($P < 0.001$).

ranase was observed in the nucleus as well as in cytoplasm [17–19], and the expression of nuclear heparanase statistically correlated with expression of a differentiation marker [19]. In in vitro assessment, however, it is difficult to determine if heparanase is correlated with differentiation in human epithelial cancer cell lines. Therefore in this study, we used breast cancer cells, which are known to express lipid droplets in their cytoplasm, which become larger and more numerous during differentiation [22,23,30], in order to facilitate the verification of the differentiation correlation. When differentiation is induced in breast cancer cells, they exhibit growth arrest, altered morphology of their cytoplasm and nuclei, expression of milk components (lipids and casein), and altered regulation of her2/neu protein expression [24–27]. Mature mammary epithelium synthesizes and secretes milk proteins and lipids in vivo [28–30].

Using pShooter/nuc-hpa vectors, we found that lipid droplets appeared in accordance with the nuclear localization of heparanase. The results showed statistically significant difference ($P < 0.001$) at 50% of transfection efficiency. The cells with overexpression of nuclear heparanase broadened displayed cytoplasm and slowed their proliferation (data not shown). These observations also support the observed correlation of nuclear heparanase with cell differentiation in cancers. In Fig. 4C, we showed that approximately 25% of transfectant cells contained lipid droplets at a transfection efficacy of 50%. However, the remaining 25% of cells expressing nuclear heparanase may be in early stages of differentiation, and we also did not count cells containing less than 10 Oil Red O-stained lipid droplets.

Induction of differentiation is one of the potent mechanisms by which some cancer therapeutic and chemopreventive agents work [31]. In human breast cancer cells, several factors have been reported to be associated with differentiation [32–39]. However, differentiation therapy using nuclear heparanase is promising for two reasons. First, in human cancer cells that express abundant heparanase in cytoplasm, the translocation of endogenous heparanase into nucleus may allow cancer cells to differentiate. Second, this translocation event may decrease the known function of heparanase in cytoplasm/cell membrane, which is to degrade the ECM thereby leading to cell invasion, metastasis, and poor prognosis. This would be very exciting for cancer therapy, although it is not yet known how heparanase translocates from cytoplasm into nucleus or how cytoplasmic heparanase can be decreased in cancer cells. Therefore, it is important to pursue the mechanism of heparanase translocation into nucleus since heparanase seems not to have a nuclear translocation signal in its sequence.

In conclusion, the results presented in this study demonstrate that overexpression of heparanase in the nucleus induces differentiation of breast cancer cells. The results also suggest that other human epithelial cancers may differentiate with nuclear heparanase. Further studies are necessary to determine how cytoplasmic heparanase can be translocated into the nucleus in the cancer cells leading to stable differentiation.

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