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# OSTEONECTIN/SPARC REGULATES CELLULAR SECRETION RATES OF FIBRONECTIN AND LAMININ EXTRACELLULAR MATRIX PROTEINS

Kyoko Kamihagi, Masahiko Katayama,* Rieko Ouchi, and Ikunoshin Kato

Cell Technology Reagent Section, Biotechnology Research Laboratories,
Takara Shuzo Co., Ltd., Seta 3-4-1, Otsu, Shiga 520-21, Japan

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**SUMMARY:** Osteonectin (OTN) has been implicated in controlling cell adhesivity onto substratum and extracellular matrix (ECM) remodeling. Significant amounts of OTN were synthesized not only by normal fibroblasts and endothelial cells, but also by HT-1080 fibrosarcoma and MG-63 osteosarcoma cells. Levels of secreted OTN were likely to be slightly elevated by the addition of exogenous placental laminin (LN), but not by supplementation of plasma fibronectin (FN). Exogenously supplemented purified bone OTN was not apparently incorporated into the ECM of the adhering cells and had no effect on cell spreading and growth, whereas secretion of type I collagen or FN in the tumor cells was moderately diminished in the presence of soluble OTN. Concentration-dependent down-regulation of cellular LN secretion appeared to be most significant, suggesting that OTN participates in regulating extracellular secretion of ECM components in the cells either with or without the ability to synthesize cellular OTN. 

Osteonectin (OTN), also termed BM40 or SPARC (secreted protein, acidic and rich in cysteine), is a component of the extracellular matrix that displays a high degree of interspecies sequence conservation (1-3). This protein is well known as a $Ca^{2+}$-binding glycoprotein, which is abundant in mineralized tissue and, to a lesser extent, in non-mineralized tissue and circulation (4, 5). It was also demonstrated that OTN modifies the interaction of cells with extracellular matrix (ECM), consequently inducing cell morphogenesis and tissue remodeling (6). Various cell lines cultured in vitro were previously found to secrete OTN, which plays an important role in their growth control, cell adhesivity, or induction of metalloprotease expression (7-9).

Fibronectin (FN) and thrombospondin are major components of ECM in normal fibroblastic and endothelial cells and their cellular production is also diminished by exogenously supplemented murine OTN, suggesting that extracellular OTN modulates ECM synthesis to achieve an optimal ratio among different components of the ECM (10). Here we report that human fibrosarcoma and osteosarcoma tumor cells in vitro secrete OTN into their ECM and culture fluids, and exogenous soluble OTN can regulate the secretion rates of FN and laminin (LN) in the cultured human cell lines secreting or not secreting OTN.

*To whom all correspondence should be addressed [Fax: (81) 775 43 2494].

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## METHODS

**Cell culture** - Established human tumor cell lines (HL-60 promyelocytic leukemia cells, MG-63 osteosarcoma cells, HT-1080 fibrosarcoma cells, and COLO 205 colon adenocarcinoma cells), normal human skin fibroblastic cells, human gizzard heart cells, and calf pulmonary aortic endothelial cells (CPAE) were all obtained from American Type Culture Collection (Rockville, MD). T-24 bladder carcinoma cells were generously provided by Dr. T. Sasaki (Cancer Research Institute, Kanazawa Univ., Kanazawa, Japan). SQ-5 human lung squamous carcinoma cells were obtained from Riken Cell Bank (Wako, Japan). Normal human placental fibroblasts were isolated from a fresh placenta provided by a pregnant Japanese woman, with her informed consent, at delivery by caesarean operation according to the published method (11). These cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum, or in the Cellgrosser serum-free cell culture medium (Sumitomo Pharmaceuticals, Osaka, Japan).
**Immunopurification of antigen** - Fetuin-rich fraction was isolated from EDTA extracts of bovine bone powder as described previously (12), and used as an immunogen to elicit murine ON1-1 monoclonal antibody (mAb) according to the standard hybridoma technology (13). Twenty milligrams of ON1-1 IgG were coupled with 10 ml of CNBr-activated agarose and the antigen was immuno-purified from the crude materials essentially according to the published method (13). Immunopurified protein was separated on a 5-20% gradient SDS-PAGE system (ATTO Co., Tokyo, Japan) without reduction and electrophoretically transferred onto the Immobilon-P membrane (Millipore, Bedford, MA). Transferred protein was immunostained with ON1-1 mAb and anti-mouse IgG rabbit antiserum conjugated with peroxidase (DAKO, Denmark). The protein band on another membrane was cut out for its N-terminal peptide sequencing analysis according to the method described previously (14). The antigen was determined to be identical with OTN (see the following section). Subsequently, another murine mAb, designated as OSN4-2, was raised against the pure antigen immunoisolated according to the above procedure.
**Platelet antigen** - Washed human platelets were isolated from citrated blood provided by healthy volunteers working in our laboratories according to the method described previously (15). Isolated platelets were suspended in 10 ml cold phosphate-buffered saline containing 2 mM EDTA, 2 mM N-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM $NaN_3$. Then, their membranes were ruptured by ultrasonication for 2 min under refrigeration. The residual membranes or debris in the extracts were completely removed by centrifugation (10, 000 *g*, 30 min) and platelet OTN was immunopurified by immobilized ON1-1 on agarose gel.
**Sandwich ELISA** - Immunoassay for soluble OTN was constructed with peroxidase-labeled OSN4-2 mAbs and immobilized ON1-1 mAbs on microtiter plates basically according to the previous procedure (16, 17). The amount of purified bovine OTN, which was utilized as a standard for assay, was estimated by absorbance at 280 nm according to the method described elsewhere (3). Human FN was purified from normal citrated plasma by gelatin-agarose chromatography and human placental LN was purchased from Takara Shuzo (Kyoto, Japan). ELISA systems for measuring fluid-phase human FN, human LN, or human procollagen type I carboxyl-terminal peptides (PIP), were purchased from Takara Shuzo. All of these sandwich ELISAs comprised peroxidase-labeled first mAbs and second mAbs immobilized on microtiter plates, and were performed as describe previously (16 -18).

## RESULTS AND DISCUSSION

We initially tried to establish mAbs reactive to bovine bone fetuin, and consequently only one mAb, ON1-1, was shown to react with the immunogen and preferentially absorbed on the microtiter plates. The ON1-1-reactive molecules in the immunogen used here were isolated by Mono-Q ion-exchange chromatography in a FPLC system (Pharmacia) using 0.02 M phosphate buffer (pH 7.5) and NaCl elution (Fig. 1). Proteins in the eluted fractions were absorbed on the microtiter plates and detected by indirect ELISA using ON1-1 mAbs, demonstrating that the immunoreactive molecule was a minor contaminant which could be eluted at relatively high ionic strength. Bovine fetuin, which exhibited an approximate molecular weight of 60 kD in SDS-PAGE, was eluted in fraction no. 9 earlier than ON1-1 antigen (Fig. 1).

Immunoblotting and protein sequencing analysis showed that this protein is identical to bovine OTN with the approximate molecular weight of 34 kD and contaminates in the crude fetuin (Fig. 2, lane 2). In the N-terminal amino acid sequencing analysis of this 34 kD protein, we identified that this protein had 11 residues on its N-terminal portion, including one indeterminant (Ala-Pro-Gln-Gln-Glu-Ala-Leu-Pro-Xaa-Glu-Thr-; Xaa indicates indeterminant), and the underlined parts matched completely with the published N-terminal sequences of the mature bovine OTN molecule (19). OTN was previously demonstrated to be present in α-granules of platelets and exposed on the cell surface during platelet activation (20, 21). Soluble OTN antigen was also purified from the supernatants of ultrasonicated platelet extracts, separated on SDS-PAGE, and analyzed by immunoblotting (Fig. 2, lane 1). We observed that ON1-1 recognized these two OTN molecules derived from bone and platelets (Fig. 2), and OSN4-2 mAb also reacted with these two forms (data not shown). In N-terminal protein sequencing, the 28 kD platelet OTN transferred onto the membrane was shown to have 10 residues, which were identical to those of bovine bone OTN obtained above or from the published sequences (22). Bone and platelet OTNs had different molecular weights of about 34 and 28 kD, respectively (Fig. 2), and this result appeared to be

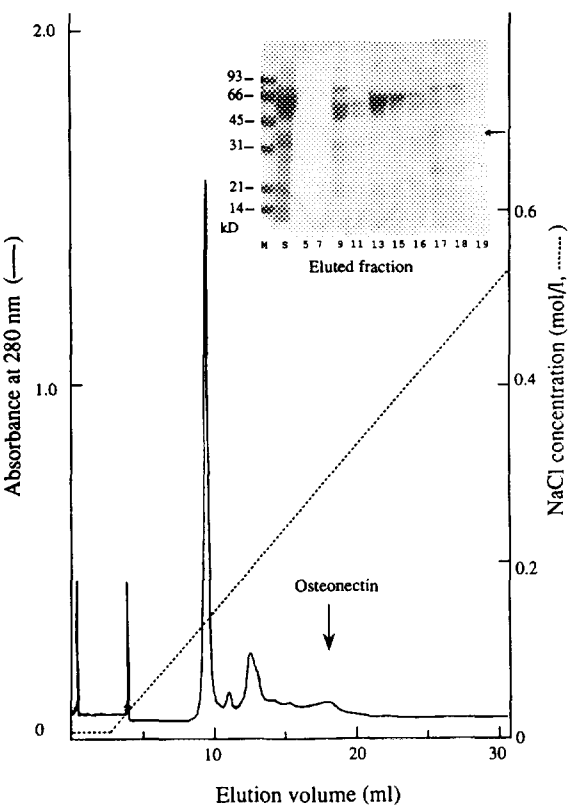


Figure 1. Separation of OTN from crude fetuin by Mono-Q ion-exchange chromatography and SDS-PAGE. Fractions of 1 ml volume were collected sequentially and monitored for absorbance at 280 nm or for the immunoreactivity on ELISA using anti-OTN mAbs. Fetuin was identified to be present in fraction no. 9. The vertical arrow indicates the elution position of OTN. Molecular weight markers (M), the crude fetuin (S), and the proteins in the eluted fractions were separated on 5-20% gradient SDS-PAGE without reduction. Numerals under the SDS-PAGE pattern indicate fraction numbers. The horizontal arrow indicates the location corresponding to OTN.

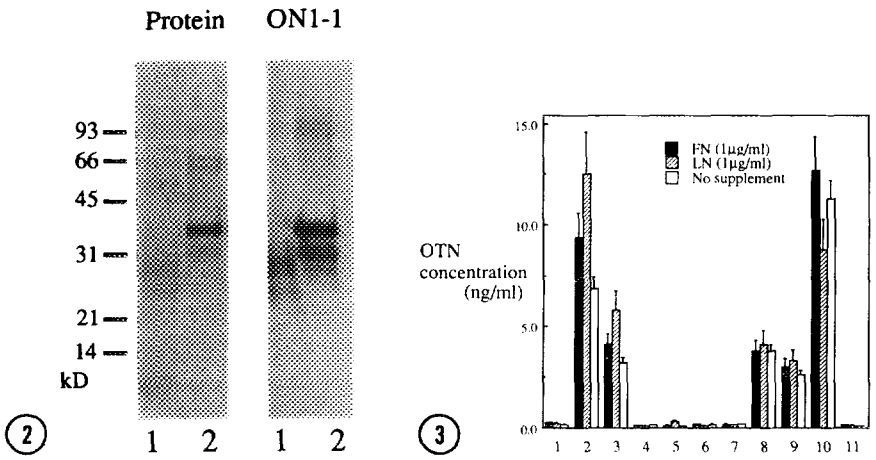


Figure 2. Western blot analysis for bone and platelet OTNs. Three micrograms of platelet OTN (lane 1) and 10 μg of OTN (lane 2) were electrophoresed, blotted onto the membrane, and subjected to Coomassie blue protein staining or immunostaining using ON1-1. Molecular weights are indicated on the left. The major stained protein was cut out for peptide sequencing analysis.

Figure 3. Levels of OTN secreted from human and bovine cell lines cultured in serum-free medium. HL-60 (1), MG-63 (2), HT-1080 (3), COLO 205 (4), T-24 (5), SQ-5 (6), gizzard heart (7), skin fibroblasts (8), placental fibroblasts (9), CPAE (10), and none (11) were cultured in serum-free medium only, in the presence of FN at the final concentration of 1 μg/ml, or in the presence of LN at the final concentration of 1 μg/ml. Fluid-phase OTN levels were measured by the mAb-based sandwich ELISA (means ± s. d. of 4 experiments).

incompatible with the previous data revealing their identical molecular weights (20). It is possible that, during the preparation of platelet extracts, some endogenous proteases might cleave the potential susceptible site on C-terminal regions of the platelet OTN molecules (23). Because the N-terminal residues of mature OTN were detected on the 28 kD fragments, the epitopes recognized by our two mAbs exist within the region of its N-terminal domain I-III (9, 23). It is evident that these two mAbs can bind equally to human and bovine OTNs.

One bovine and nine human cell lines were cultured simultaneously for 6 days in the conventional serum-free medium (approximate 5 X $10^7$ cells per 10 ml medium in a 9-cm-diameter culture dish) containing 1 μg/ml human plasma FN, 1 μg/ml human LN, or no supplement, at the final concentration. Soluble OTN levels in the culture supernatants were measured by the ELISAs. Significant amounts of soluble OTN were detected in the culture supernatants of normal fibroblasts and CPAE. Among the tumor cell lines, MG-63 and HT-1080 cells secreted soluble OTN (Fig. 3). No OTN antigen was detected in the other myelocytic, epithelial or epithelial-like cell cultures. Supplemented human LN slightly increased OTN secretion from normal fibroblasts, and more significantly increased those from MG-63 and HT-1080. In contrast, however, the levels of soluble OTN released from CPAE were diminished (Fig. 3). Thus, it is possible that soluble LN or insoluble LN in ECM can potentially regulate cellular OTN synthesis.

Extracellular OTN is closely related with the interactions of a cell with its surrounding ECM, resulting in the regulation of cell behavior and tissue architecture (6). Here, we examined the effect of soluble bovine bone OTN on cellular synthesis of ECM proteins. Purified bovine OTN was supplemented into these cell cultures according to the above supplementation procedure.

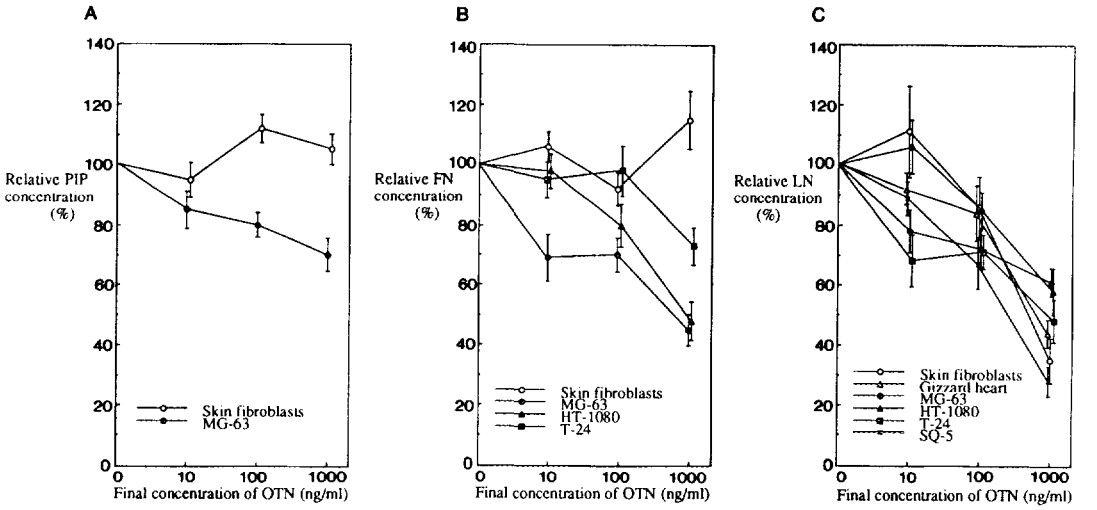


Figure 4. Effects of supplemented OTN on cellular secretion rates of glycoprotein including PIP (A), FN (B), and LN (C). Human cell lines were cultured in the serum-free medium containing bovine bone OTN at final concentrations of 10, 100, and 1000 ng/ml for 6 days. Relative glycoprotein secretion was expressed as percent of control value obtained in the culture with no supplemented OTN (means ± s.d. of 4 determinants).

OTN retained its solubility in a serum-free medium for 6 days, because OTN levels introduced at the onset were completely recovered after the cultivation. Type I collagen (Col I) is the most abundant collagen species in the human body. Its synthesis involves the removal of large domains from both ends of a precursor molecule, and the protein released from the C-terminal end, known as PIP, reflects Col I synthesis activity (24). The removal of PIP is a necessary prerequisite for Col I fibril formation, and thus PIP cannot remain attached to the collagen and is mostly released into the circulation in animals (25). Fibroblasts and MG-63 cells can produce Col I and release a significant amount of PIP; however, PIP was undetected in the culture media of the other cell lines used here, demonstrating that they have no ability to produce Col I. Col I synthesis in MG-63 was found to be moderately inhibited by exogenous soluble OTN in a dose-dependent manner (Fig. 4-A). Fibroblasts and some of the tumor cell lines (HT-1080, MG-63, and T-24) apparently express cellular FN. FN synthesis by the three tumor cell lines decreased significantly in the cultures supplemented with a large amount of OTN (Fig. 4-B). In contrast, the presence of OTN did not alter the secretion of Col I and FN in normal skin fibroblasts (Figs. 4-A and 4-B). Similar results were observed in the experiments using cultured placental fibroblasts (data not shown). Interestingly, cellular production of LN was found to be markedly down-regulated by exogenous OTN in a dose-dependent manner in all of the cultured cells utilized in the present study (Fig. 4-C). T-24 and SQ-5 are epithelial-like tumor cell lines able to secrete FN or LN, without the ability of cellular OTN production. Their cellular production rates of ECM components were drastically diminished, indicating that exogenous OTN plays a key role in the modulation of ECM components. Surprisingly, changes of cell shape and growth rate were negligible in all of the cells used here in the presence of exogenous bone OTN (data not shown). Murine OTN isolated from PYS-2 cells has been usually used to interpret biological effects of OTN including anti-adhesive funcion and growth control (3, 6, 7). It was somehow assumed that OTN molecules from different species or tissues may have some dissimilar biological activities on cultured cells.

Soluble OTN circulates in normal human circulation (5). OTN is also detected abundantly in resting platelets, which may release significant amounts of OTN into circulation under activation (20, 21). It is hypothesized that alternative remodeling of ECM components can be induced by increased OTN in injured intravascular sites or in other affected areas such as the connective tissues around progressive tumor, particularly modulating extracellular LN secretion. We preliminarily observed that cellular OTN secretion by normal fibroblasts or CPAE was drastically enhanced in fetal calf serum-supplemented culture, as compared with that under the serum-free condition (data not shown). If there is a possible mechanism that some growth factors in fetal serum can directly or indirectly modulate OTN production rates in fibroblasts and endothelial cells, then the in vivo biological effect of soluble OTN released from these cells may be considered to be physiologically important on the ECM formation in their surrounding tissues.

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