Med Mol Morphol (2009) 42:155–161
DOI 10.1007/s00795-009-0459-6

ORIGINAL PAPER

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The immunohistochemical expression profile of osteopontin in normal human tissues using two site-specific antibodies reveals a wide distribution of positive cells and extensive expression in the central and peripheral nervous systems

Received: March 9, 2009 / Accepted: June 3, 2009

Abstract To elucidate the cellular distribution of osteopontin (OPN) in normal human tissues, we undertook immunohistochemistry using two site-specific OPN antibodies. The 10A16 monoclonal antibody was raised against the amino acid sequence just downstream of the thrombin cleavage site, while the O-17 polyclonal antibody was raised against the N-terminal peptide. Each antibody has been confirmed previously to react with both whole OPN and its relevant fragments. The expression pattern for these two antibodies was similar in distribution. In addition, we also identified expression in Ebner's gland, type II pneumocytes, Kupffer cells, cells of the endocrine organs, anterior lens capsule and ciliary body, synovial type A cells, mesothelia, adipocytes, and mast cells. Neurons and glia in the central nervous system and spinal cord, cranial and peripheral nerve sheaths, ganglion cells in the sympathetic ganglion, intestinal plexuses, retina, and choroid plexus also regularly exhibited OPN positivity. Testicular germ cells, pancreatic exocrine cells, and follicular dendritic cells reacted with 10A16 only, whereas lutein cells and taste bud cells exhibited O-17 reactivity alone. These minor differences were hypothesized to reflect the state of OPN in the cells; that is, whether OPN was in its whole molecule or fragmented form. In conclusion, we demonstrate that OPN is widely distributed in normal human cells, particularly those comprising the central and peripheral nervous systems.

Key words Osteopontin · Immunohistochemistry · Human Tissue · Expression profile

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Introduction

Osteopontin (OPN) is an acidic, secreted protein that has recently been classified as a member of the small integrin-binding, N-linked glycoprotein (SIBLING) family.[1] Starting from the N-terminus, OPN contains a motif with 7–10 consecutive aspartic acid residues depending on the species, the cell-binding sequence argininylglycylaspartic acid (RGD), a thrombin cleavage site (6 amino acid residues downstream of the RGD motif), a cryptic epitope (SVVYGLR in humans) postulated to be involved in angiogenesis,[2] a calcium-binding region, and a hyaluronic acid receptor (CD44)-binding domain.[3,4] Despite this peculiarity of structure and its resulting fragility, OPN has been shown to exhibit numerous functions in vitro and in vivo, including important roles in calcium binding, chemotaxis, cell adhesion, cell signaling, cell proliferation, and cell survival. Several in vitro and a few in vivo studies have revealed that the thrombin-cleaved OPN fragments in general possess higher activity than the full-length form and that the thrombin-cleaved fragment also gains a cell-interacting domain.[5]

OPN was initially found to be expressed predominantly in bone, kidney, and body fluids. More recent immunohistochemical studies have since shown its expression in the luminal surfaces of human epithelial cells comprising several tissues and organs such as the gallbladder, lung, mammary gland, gastrointestinal, urinary and reproductive tracts, and salivary and sweat glands.[6–12] In addition, it is known that OPN is able to undergo posttranslational modification depending on the tissue site or factors that regulate its expression.[13,14] The immunohistochemical localization of OPN in cells and tissues, therefore, appears to differ according to the antibodies used and the rather fragile nature of OPN. In the present study, we examined the precise cellular localization of OPN in normal human tissues using site-specific OPN antibodies. Antibody 10A16 was directed against the region just downstream of the thrombin cleavage site, while O-17 recognized the N-terminal peptide of OPN.

Materials and methods

Sample tissues

Ethical approval was obtained from the Fukushima Medical University local research ethics committee, and informed written consent was obtained from patients or their parents for the pathological examination of tissues. Normal human tissues were obtained from autopsy cases. An eye resected because of retinoblastoma in a 1-year-old infant and placental tissues and umbilical cord at 20 weeks of gestation following spontaneous abortion were also used. All specimens were fixed in 10% buffered formalin and embedded in paraffin.

Immunohistochemistry

Antigenic amino acid sequences and localization of the sequences in human OPN have been described previously.[15] Using these amino acid sequences, mouse monoclonal OPN antibody 10A16 and rabbit polyclonal OPN antibody O-17 were produced by IBL (Takasaki, Gunma, Japan). Each antibody has been previously confirmed to react with both the whole OPN and its relevant fragments using Western blotting (Kon et al.[15]). 10A16 antibodies were used at 0.5 μg/ml IgG and O-17 at 1 μg/ml IgG. For immunohistochemistry, 5-μm tissue sections were deparaffinized, rinsed with phosphate-buffered saline (PBS), and endogenous peroxidase activity blocked with 0.03% hydrogen peroxide in methanol for 20 min. The sections were then incubated in 5% skim milk (Yukijirushi, Sapporo, Japan) for 30 min, followed by incubation with primary antibody and then either biotinylated goat antirabbit or biotinylated rabbit antimouse immunoglobulins (Histofine SAB-PO [R] or [M] kit; Nichirei, Tokyo, Japan). After washing, avidin-horseradish peroxidase (Histofine SAB-PO kit) was applied to the slides. Finally, 3,3'-diaminobenzidine tetrahydrochloride (Dojin, Kumamoto, Japan) was used as the chromogen, and cellular nuclei were counterstained with 1% methyl green or hematoxylin. The immunostained sections of eye and midbrain (substantia nigra) were counterstained with Giemsa to distinguish the reaction products from melanin pigments. Bone samples were decalcified before preparation of the tissue sections.

Evaluation of immunohistochemical staining

When positive staining of the cells was absent, the cell population was given a score of 0 (none). When the positive cells comprised less than 5% of the total cell population, a score of 1 (low) was given. Similarly, when the frequency was 5%–25%, the score given was 2 (moderate), and for more than 25%, a score of 3 was given (high). Thus, staining intensity was scored as 0 (none), 1 (weak), 2 (moderate), or 3 (strong). The combined scores finally used were as follows: less than 2, slight (+); 3–4, moderate (++); and 5–6, high (+++).

Results

Similar to previously published reports, many kinds of epithelial cells including those comprising the gastrointestinal tract, kidney (loop of Henle and distal convoluted tubules), urinary bladder, uterine glands, breast, lung (bronchus), gallbladder, bone, and macrophages were labeled using both the 10A16 and O-17 antibodies. We also found that a number of additional cell populations expressed OPN positivity in the present study, such as Ebner's gland cells of the tongue (Fig. 1A), Kupffer cells of the liver (Fig. 1B), islet cells of the pancreas (Fig. 1C), type II alveolar cells of the lung (Fig. 1D), Leydig cells of the testis, seminal vesicle epithelia, Hofbauer cells of the placenta, follicular cells of the thyroid (Fig. 1E), a subset of adenohypophyseal cells (Fig. 1F), concrements and ganglion cells of the neurohypophysis, type A cells of synovium, sarcolemmas of skeletal muscle, mesothelial cells, fat cells, mast cells, eosinophils in the bone marrow, and neutrophils that had infiltrated the tissues. Megakaryocytes and erythroblasts were not labeled by either of the antibodies. Stromal fibroblastic cells in skin, uterus, ovary, paraoptic nerve, and umbilical cord also expressed OPN. In the small intestine, basal cells of glands and macrophages located among the apical epithelial cells of the intestinal glands appeared intensely positive. Infrequent immature chondrocytes in the vertebrae (Fig. 1G-1) and osteocytes (Fig. 1G-2) expressed OPN. In addition, widespread positive cells were observed in the central and peripheral nervous systems, particularly the neurons of the cerebral cortices (mainly of the III–IV layers in the frontal, parietal, lateral, and occipital lobes) (Fig. 1H), the neurons in the basal ganglia, thalamus, ammon, midbrain (substantia nigra) and medulla oblongata, glia (most likely astroglia) (Fig. 1I), the neurons of the spinal cord (Fig. 1I), ganglion cells of the retina (Fig. 1J), choroid plexus cells, myelin sheaths of the peripheral and X, XI, and XII cranial nerves (Fig. 1K), and the ganglion cells of the sympathetic ganglion (Fig. 1L), Auerbach's plexus (Fig. 1M), and Meissner's plexus. The pineal body contained only a few positive pinealocytes, but intensely positive psammoma bodies. Inner nonpigmented layer cells of the ciliary body and cells of the anterior lens capsule expressed OPN. Many of the OPN-positive neurons in the cerebral cortices revealed both cytoplasmic and axonal positivity. Several nerve fibers were also labeled (Fig. 1H).

We also revealed numerous differences in OPN staining when using the 10A19 and O-17 antibodies. Basal layer cells of the skin demonstrated an absence of staining, while the keratinocytes demonstrated more intense staining when using the 10A16 antibody in comparison to the O17 antibody (Fig. 2B). In the gastrointestinal tract, 10A16 labeled mucus present in the goblet cells while O-17 did not; this was also the case for the cervical glands of the uterus, which were positive following incubation with antibody 10A16 (Fig. 2C) but were negative when using O-17 (Fig. 2D). Secreta of prostatic glands and the breast were more intensely stained when using 10A16 compared with that following the use of O-17. Purkinje cells of the cerebellum

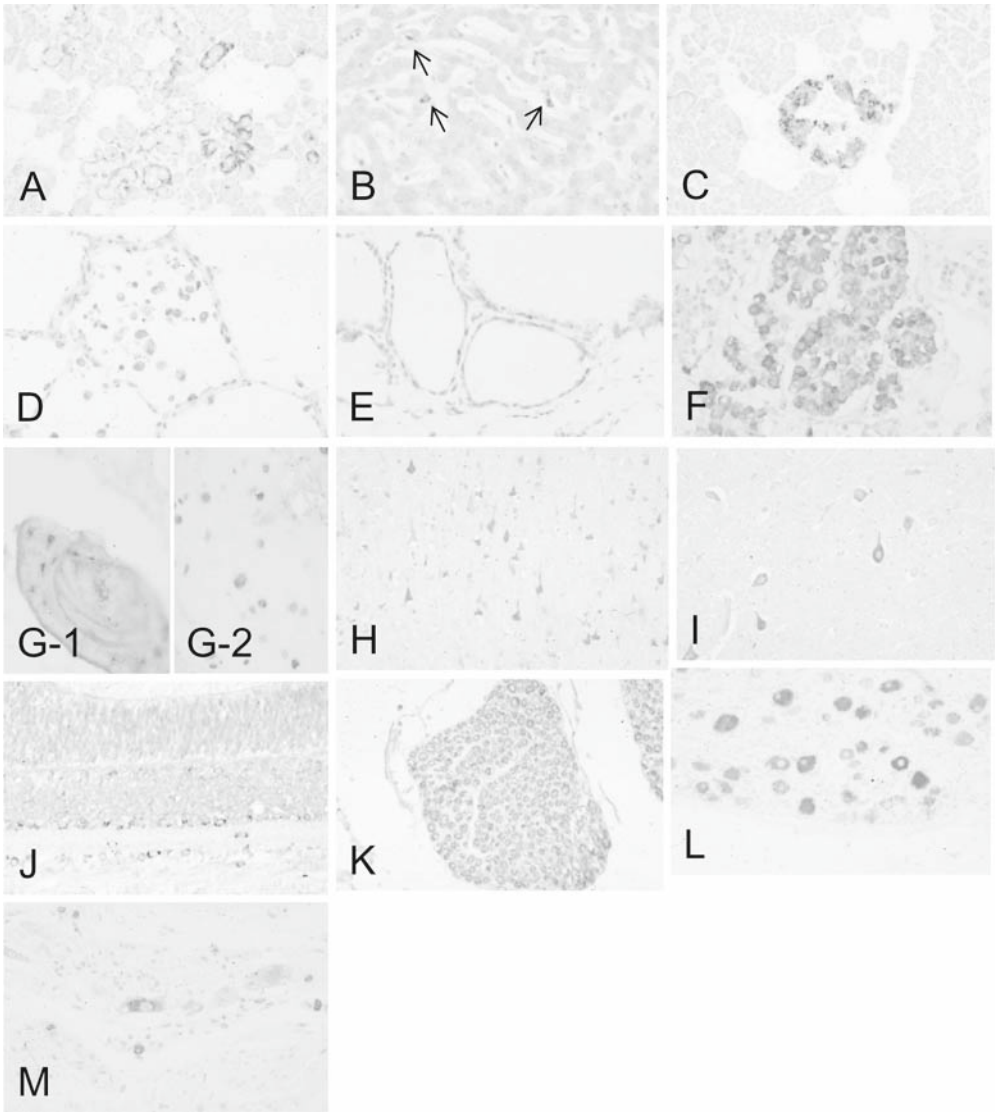

Fig. 1. Numerous cell populations were labeled using both 10A16 and O-17 antibodies: Ebner's gland of tongue (**A**), Kupffer cells of liver (**B**, *arrows*), pancreatic islet cells (**C**), type II alveolar cells of the lung and macrophages (**D**), thyroid follicular cells (**E**), anterior pituitary cells (**F**), osteocytes (**G-1**), round immature chondrocytes (**G-2**), larger neurons mainly in the IIIrd to VIth layer of the cerebral cortex (frontal lobe, **H**), neurons and glia in the cervical spinal cord (**I**), ganglion cells and inner nuclear layer cells of an infant retina (**J**), XIth cranial nerve (**K**), ganglion cells of the sympathetic ganglion (**L**), and Auerbach's plexus (**M**)

also demonstrated intense reactivity to 10A16 (Fig. 2E) when compared with that of O-17 (Fig. 2F). In contrast, colloids of the thyroid exhibited a weaker OPN reactivity following 10A16 incubation than O-17. In addition, several cells were found to react only with 10A16. These cells included pancreatic exocrine cells (Fig. 2G,H) and germ cells including spermatozoa and follicular dendritic cells located in the germinal centers. In contrast, lutein cells (Fig. 2I) and taste bud cells exhibited O-17-reactivity alone. This pattern of staining, however, was not as intense.

The precise OPN expression pattern in normal human tissues is summarized in Tables 1, 2, and 3.

Discussion

OPN is known to be expressed in many kinds of normal human cells. In the current study, we confirmed the OPN expression pattern using two site-specific OPN antibodies, termed 10A16 and O-17, in cells previously described in the literature to contain OPN protein or OPN mRNA. These cells included the brain, kidney, decidua, and placenta.[16] endometrial glands and decidual cells,[17–19] gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung, mammary gland, sweat and salivary glands,[6,8,11,20] macrophages and cytotrophoblasts,[21–24] liver, lymph node, spleen and testis,[25] renal distal tubules and Henle loops,[26,27] prostate,[28] eccrine sweat gland duct,[29] keratinocytes, and hair follicles and sebaceous glands.[30] In the present study, we also found that a number of cells that have not been previously described to express OPN were labeled using both antibodies. These cells included inner nonpigmented layer cells of the ciliary body, cells of the anterior lens capsule, Ebner's gland cells of the tongue, Kupffer cells, islet cells of the pancreas, type II pneumocytes, seminal vesicle epithelia, Hofbauer cells of the placenta, follicular cells of the thyroid, adenohypophyseal cells, sarcolemma of skeletal muscle, type A synovial cells, immature chondrocytes, osteocytes, mesothelial cells, fat cells, neutrophils, mast cells, eosinophils, and fibroblastic stromal cells in the skin, uterus, ovary, umbilical cord, and paraoptic nerve.

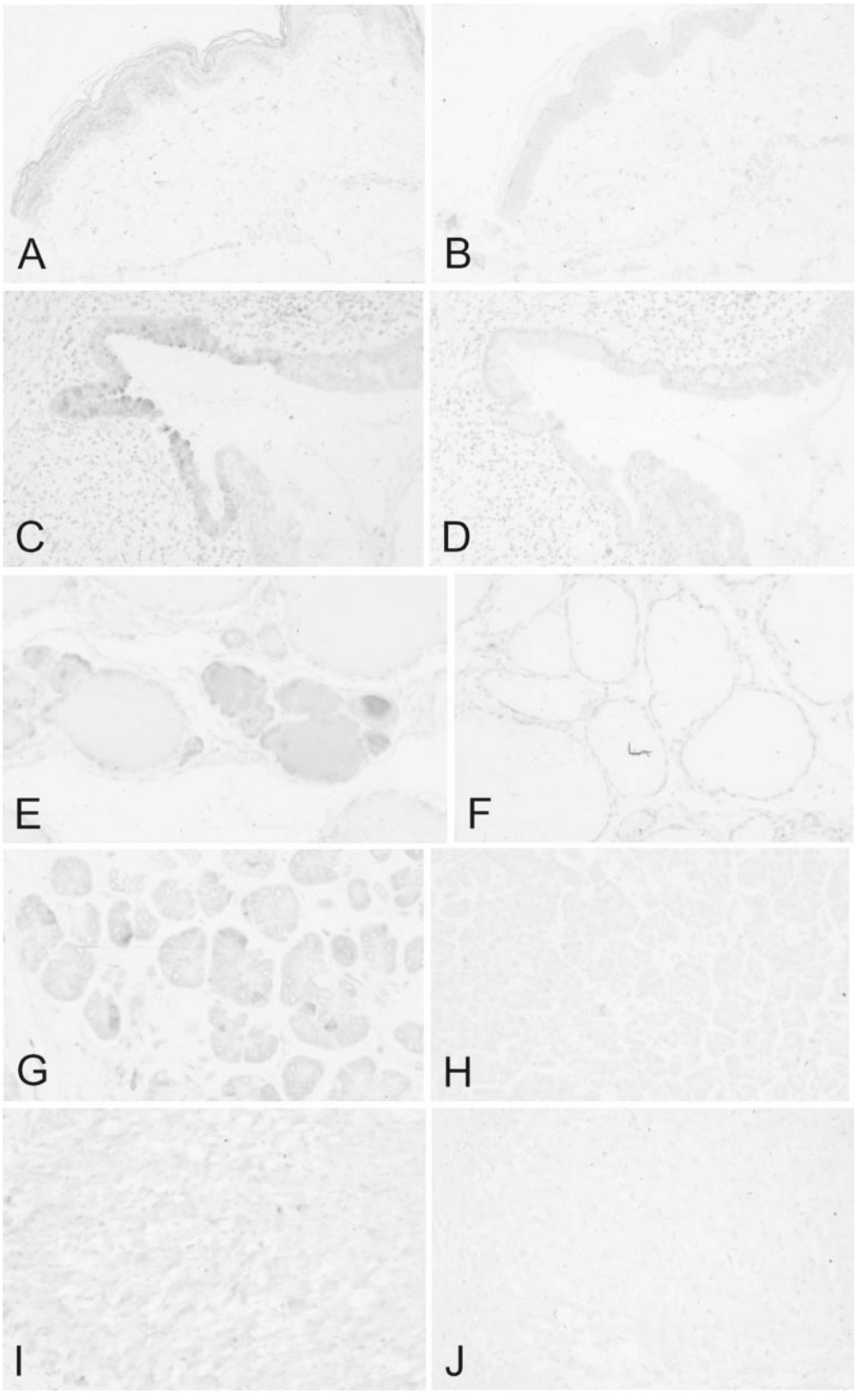

Fig. 2. Different staining intensity was observed for the 10A16 and O-17 antibodies. Skin squamous epithelia was intensely stained using 10A16 (**A**) and weakly stained using O-17 (**B**); mucus of uterine cervical gland using 10A16 was positive (**C**) and negative using O-17 (**D**); expression was observed in the small thyroid colloids using O-17 (**E**) but not 10A16 (**F**). In addition, some cells revealed either 10A16 or O-17 expression alone. A subset of pancreatic exocrine cells (**G**) revealed 10A16 reactivity, but not O-17 reactivity (**H**). In contrast, lutein cells of the ovary (**I**) show O-17 reactivity but no reactivity to 10A16 (**J**)

Expression of OPN in human endocrine cells has not been described previously; however, endocrine expression has been suggested, given reports that OPN is detected in rat anterior pituitary gonadotropes[31] and OPN in somatostatin cells of the human pancreas islets.[32] Kupffer cells and macrophages in the normal rat liver have been shown to exhibit minimal expression of OPN mRNA, while cells from the liver of *Propionibacterium acnes*-treated rats,[33] or

Table 1. Expression of osteopontin in normal human tissues

Tissue or cells	10A16	O-17
Skin		
Keratinocytes	(+++)	(+)
Hair follicle cells	(+)	(+)
Duct cells of sweat glands	(+)	(+)
Sebaceous glands cells	(+)	(+)
Tongue		
Squamous epithelia	(+)	(+)
Ebner's glands	(+)	(+)
Taste buds	(−)	(+)
Parotid		
Serous glands	(+)	(−)
Mucus glands	(+)	(−)
Esophagus		
Squamous epithelia	(+)	(−)
Mucus glands	(+)	(−)
Stomach		
Mucus neck cells	(+)	(+)
Foveolar cells	(+)	(−)
Chief cells/mucus	(+)/(+)	(+)/(−)
Duodenum		
Epithelial cells/mucus	(+)/(+)	(−)/(−)
Jejunum, ileum		
Basal cells/mucus	(++)/(+)	(++)/(−)
Colorectum and appendix		
Goblet cells/mucus	(+)/(+)	(+)/(−)
Liver		
Bile duct epithelia	(+)	(+)
Kupffer cells	(+)	(+)
Gallbladder		
Epithelia	(+++)	(+++)
Pancreas		
Exocrine cells	(+)	(−)
Islet cells	(+)	(++)
Lung		
Bronchial epithelia	(+)	(+)
Alveolar Møs	(+++)	(+++)
Type II alveolar cells	(+)	(+)
Kidney		
Proximal tubules	(+)	(−)
Henle's loop	(+)	(+)
Distal tubules	(+)	(+)
Urinary bladder		
Epithelia	(+)	(+)
Ureter		
Epithelia	(++)	(+)
Prostate		
Glands/secreta	(+)/(+++)	(+)/(+)
Testis		
Germ cells	(+)/(+)	(−)/(−)
Leydig cells	(+)	(+)

(+++), high expression; (++), moderate expression; (+), low expression; (−), negative; (−)-(+), occasional positive expression

Table 2. Expression of osteopontin in normal human tissues

Tissue or cells	10A16	O-17
Seminal vesicle		
Epithelia	(+++)	(+)
Uterus		
Endometrial glands	(+)	(++)
Cervical glands	(+)	(−)
Stromal cells	(+)	(+)
Ovary		
Lutein cells	(−)	(++)
Stromal cells	(+)	(+)
Granulosa/theca cells	(−)	(+)
Oviduct		
Epithelia	(+)	(−)
Placenta		
Trophoblasts	(+)	(+)
Hofbauer cells	(+++)	(+++)
Calcified foci	(+++)	(+++)
Decidua		
Decidual cells	(+)	(+)
Breast		
Glands/secreta	(+++)/(+++)	(+)/(+)
Duct cells	(+)	(+)
Umbilical cord		
Stromal cells	(+)	(+)
Pituitary gland		
Anterior lobe	(+)	(+)
Posterior lobe	(+)	(+)
Thyroid		
Glands/colloid	(+)/(+)	(+)/(+)
Thymus		
Hassall body epithelia	(−)	(+)
Calcified foci	(+++)	(+)
Lymph node		
Møs	(+)	(+)
Plasma cells	(−)	(+)
FDCs	(+)	(−)
GCs	(+)	(−)
Spleen		
Parafollicular cells	(+)	(−)
GCs	(+)	(−)
Cartilage		
Chondrocytes	(+)	(+)
Bone		
Osteocytes	(+)	(+)
Synovium		
Type A cells	(++)	(+)
Bone marrow cells		
Granulocytic series	(+)	(−)
Eosinophils	(+)	(+)

FDCs, follicular dendritic cells; GCs, germinal centers of lymph follicle

from rats subjected to carbon tetrachloride intoxication,[34] reveal marked OPN expression. In our study, a subset of human Kupffer cells exhibited OPN expression without noticeable hepatic stimulation. It has been documented previously that murine mast cells produce OPN in culture,[35] and thus mast cells, as well as eosinophils, revealed expression of OPN. OPN expression in these cell populations may have been induced in part by any stimuli or stresses during the period of disease or during surgery. Such induction appears likely, given that expression of OPN is upregulated with ease by various agents or conditions such as hormones (cardiomyocytes and microvascular endothelial cells by glucocorticoid,[36] trophoblasts by progesterone,[37] thyroid by thyrotropin and iodide[38]), inflammation (pancreas),[39] vitamin D (kidney[27] and endometrium[40]), calcium, parathormone, and cytokines (kidney[27]), hypoxia (vascular smooth muscle cells[41]), or chronic heart failure (cardiomyocytes[42]).

As described in the Results section of this article, minor differences in reactivity were observed for the 10A16 and O-17 antibodies. Potential causes of these discrepancies in detection is not clear; however, it is likely that several cells or cell products may contain only OPN fragments downstream of the thrombin cleavage site and vice versa, or that

Table 3. Expression of osteopontin in normal human tissues

Tissue or cells	10A16	O-17
Cerebrum (frontal, parietal, lateral, and occipital lobes; thalamus; basal ganglia; ammon; substantia nigra)		
Neurons	(++)~(+++)	(++)~(+++)
Glias	(+)~(+++)	(+)~(+++)
Fibers	(+)	(+)
Cerebellum		
Purkinje cells	(+)	(+)
Neurons in granular cell layer	(+)	(+)
Glias	(+)	(+)
Fibers	(+)	(+)
Medulla oblongata		
Neurons	(+++)	(+++)
Glias	(+)	(+++)
Fibers	(+)	(+)
Spinal cord (cervical)		
Neurons (both horns)	(++)	(+)
Glias	(++)	(+)
Fibers	(+)	(+)
Pineal body		
Pinealocytes	(+)	(+)
Psammoma bodies	(+++)	(++)
Choroid plexus		
Lining cells	(+)	(+)
Cranial nerves (x, xi, xii)		
Nerve sheaths	(+++)	(+++)
Peripheral nerves		
Nerve sheaths	(+)	(+)
Optic nerve		
Glias	(−)	(+)
Sympathetic ganglion		
Neurons	(+++)	(+++)
Schwann cells	(+)	(+)
Auerbach's plexus		
Ganglion cells	(+++)	(+++)
Meissner's plexus		
Ganglion cells	(+)	(+)
Retina		
Ganglion cells	(+)	(+)
Lens:		
Anterior lens capsule	(+)	(+)
Ciliary body		
Inner nonpigmented layer	(+)	(+)
Skeletal muscle		
Sarcolemmas	(+)	(+)
Vascular or intestinal	(+)	(+)
Smooth muscle cells:		
Mesothelial cells:	(+)	(+)
Fat cells	(+)	(+)
Endothelial cells of blood vessels:	(+)	(+)
Neutrophils (infiltrated in tissues)	(+++)	(+)
Mast cells	(+++)	(+++)

cleavage may occur during stressful stages of disease development.

Although Butler has reported that OPN is synthesized in the brain,[16] the precise cellular localization and distribution of OPN in the nervous system has not been previously described. In the present study, we observed extensive expression in the central and peripheral nervous systems. Many neurons in the IIIrd to IVth layer of the cerebral cortices and in thalamus, ammon, substantia nigra, medulla oblongata, and spinal cord revealed reactivity to both antibodies. Moreover, OPN was expressed in choroid plexus, myelin sheaths of cranial and peripheral nerves, ganglion cells of retina, sympathetic ganglion, Auerbach's plexus and Meissner's plexus, and Schwann cells. To the best of our knowledge, expression of OPN has been described only in substantia nigra neurons,[43,44] myelinating Schwann cells,[45] ganglion cells of the bowel wall,[6] neurons in rat spinal cord,[46] and ganglion cells of rat retina.[47,48] In addition, during conditions of neurodegeneration, OPN has been shown to be highly expressed in neurons of amnion in Alzheimer's disease,[49] but is decreased in substantia nigra neurons, and is increased in the microglia in Parkinson's disease.[44] The biological significance of OPN in the nervous system remains to be precisely elucidated under physiological and pathological conditions.

In conclusion, we demonstrate that OPN is widely expressed in normal human cells including the central and peripheral nervous systems, and more than 18 cell types are located in various organs. Although the 10A19 monoclonal OPN antibody was found to exhibit slight advantages over the O-17 polyclonal OPN antibody in the labeling of OPN-positive cells, the use of both antibodies in combination is recommended to identify OPN expression in human tissues and cells.

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