The secreted phosphoprotein osteopontin (OPN), when immobilized on a surface, supports cell adhesion, prevents apoptosis of endothelial cells, and is a ligand for the αvβ3 integrin, which is important in endothelial cell biology and neovascularization. OPN synthesized by tumor cells stimulates tumor growth, but the mechanism by which the protein acts remains unclear. One possibility, therefore, is that OPN may exert its effects on tumor growth by enhancing angiogenesis. While OPN is found at high levels in bone, where it is a component of the mineralized matrix, we have asked here whether OPN present in tumors is similarly extracellular matrix associated. We have shown that OPN is detectable in tumor extracts and in serum of tumor-bearing mice, and that the protein in tumors and in serum can be synthesized by both tumor and the host cells. Biochemical fractionation of tumor tissue confirmed that there is little if any association of OPN with the insoluble fraction. Immunohistochemical analysis of murine mammary tumors shows no co-localization of OPN with the extracellular matrix, identified by laminin staining. Ras-transformed cells in culture produce abundant OPN, however, the protein was found to be associated with the cell fraction but not with the matrix fraction. An enzyme-linked immunosorbent assay was used to demonstrate that OPN in conditioned medium from these cells fails to associate with extracellular matrix components, including laminin and fibronectin, in vitro. Recombinant OPN (GST-OPN) when coated onto a plastic surface can support human umbilical vein endothelial cell adhesion, suppressing apoptosis and allowing cell cycle progression, at concentrations from 1 to 50 μg/ml. Soluble GST-OPN in the same concentration range has no effect on HUVECs held in suspension. Thus, we conclude that OPN associated with tumors is primarily soluble, and that soluble OPN can neither support endothelial cell proliferation nor prevent apoptosis of these cells in the absence of adhesion.
the situation in tumors: if OPN, which is expressed at quite high levels in many tumor types, is functioning as a cell adhesion molecule it should be immobilized in the matrix. In the work presented here, we have used a variety of techniques to localize OPN in tumor tissues and cells, and conclude that OPN made by tumors is primarily, if not exclusively, soluble.

MATERIALS AND METHODS

Reagents—Laminin (Sigma), fibronectin (In Vitrogen), collagen I and matrigel (Collaborative Research/BD) were obtained from commercial sources, while recombinant GST-OPN was isolated as described (31). mOPN was reconstituted in 10% DMSO. After washings, cell or ECM extracts were separated by using DEAE-Sepharose chromatography. Antibodies to laminin and fibronectin were obtained from Sigma. Antibodies to OPN were: goat anti-rat OPN antibody OP199 (28) or rabbit anti-human OPN antibody LF 123 (32); both these antibodies were used at a dilution of 1:1000; they both react with mouse OPN and were used interchangeably.

Tumors and Tumor Extracts—The tumors used here were derived from two different experimental systems. Mammary tumors arising spontaneously in MMTV-c-myc/MMTV-v-Ha-ras transgenic mice, either wild type or OPN-deficient (34), were used for analysis of extra-cellular matrix. For the analysis of OPN accumulation in sera and tumors in hosts of different genotypes, tumors were induced by injection of ras-transformed 3T3 cells as described (35) into either wild type or OPN-deficient mice. In all cases, mice were sacrificed by exsanguination, and tumors were removed and immediately flash frozen on liquid nitrogen. Powdered frozen tissue was stored at −70 °C until use. Tumor extracts were made by resuspending the powdered tissue in RIPA Tris/Triton, and sonication. The suspension was centrifuged (15,000 g/10 min) and the supernatant retained. The wells were then washed with water, and the matrix containing wells, and some of the wells containing directly coated conditioned medium were fixed with 4% paraformaldehyde for 30 min at room temperature. Following additional washes, OPN was detected with affinity purified polyclonal antiserum OPN 1:3000 diluted in PBS at room temperature. Tumors arising in both wild type and OPN−/− mice were used (34).

Immunohistochemistry—Serial sections from myc/ras mammary tumors (38) (fixed in methacarn and embedded in paraffin) were rehydrated and endogenous peroxidases blocked in 3% H2O2 in methanol. Tumors arising in both wild type and OPN−/− mice were used (34). Sections were blocked with 5% normal goat serum for 30 min. Primary antibodies were diluted in 1% BSA, 1% goat serum, and incubated with tissue sections for 1 h or overnight. Affinity purified polyclonal anti-rat OPN (1:1000) was biotinylated with the DAKO ARK system before application to the tissue. Rabbit anti-laminin was from Sigma, was used at 1:300 and detected with biotinylated goat anti-rabbit IgG.

Table counts in the wells.

ELISA—Wells of 96-well plates were coated with matrix components at 200 g/ml in PBS overnight at 4 °C. These wells were blocked the next day with 2% BSA. Conditioned medium from either wild type or OPN-deficient ras-transformed cells was then added to the coated well and incubated at room temperature for 2 h. The same conditioned medium, after dilutions as needed, was applied directly to uncoated wells. All wells were then washed with water, and the matrix containing wells, and some of the wells containing directly coated conditioned medium were fixed with 4% paraformaldehyde for 30 min at room temperature. Following additional washes, OPN was detected with affinity purified polyclonal antiserum OPN 1:3000 diluted in PBS at room temperature. Tumors arising in both wild type and OPN−/− mice were used (34). All sections were reacted with the ABC reagent (Vector ABC Elite) prior to visualization of the antibody reactivity by staining with diaminobenzidine (Sigma Fast DAB). Sections were lightly counterstained with hematoxylin and mounted in permount. Control sections were incubated without primary antibody.

Confocal Microscopy—Paraformaldehyde sections from myc/ras mammary tumors (38) were rehydrated and subjected to antigen retrieval (0.01% citrate, pH 6.0, 100 °C, 10 min). Sections were blocked with 5% normal goat serum (1:1000 in PBS) for 1–2 h. Primary antibodies were diluted in 1% BSA, 1% goat serum, and incubated with tissue sections overnight. Affinity purified monoclonal antibody 2A1 at 0.18 μg/ml was biotinylated with the DAKO ARK system before application to the tissue, and was detected with rhodamine-avidin D (Vector Labs, 1:300 dilution) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Jackson). Rhodamine and fluorescein isothiocyanate signals were collected and a single optical section is shown. Cells were blocked with 5% normal goat serum (1:1000 in PBS) before PDL 15. Prior to experiments, confluent cells were incubated with 10% fetal bovine serum for 1 h, then 1 μg/ml heparin, and used without further washing. For apoptosis, cells were detached with trypsin, washed with PBS, and used without further washing. Control experiments showed that this protocol recovered all the trichloroacetic acid-soluble counts in the wells.

For adhesion assays, exponentially growing cells in complete medium were harvested with trypsin, resuspended in complete medium, and washed in Dulbecco’s modified Eagle’s medium containing 3% fetal bovine serum on 35-mm wells previously coated with 0.3 mg/ml collagen I in 20 mM acetic acid or with 0.7 mg/ml Hoescht 33342. Floating and adherent cells were collected, fixed, and counted under fluorescence illumination. For [3H]thymidine incorporation, cells were incubated overnight with growth factors (10 ng/ml FGF plus 5 units/ml heparin), then 2 μCi/ml [3H]thymidine were added and the cells were incubated for an additional 5–6 h. Cells were washed, fixed in 7% trichloroacetic acid, then solubilized in 0.5 N NaOH, 0.5% SDS and counted. Control experiments showed that this protocol recovered all the trichloroacetic acid-soluble counts in the wells.

2 A. J. Kowalski, S. R. Ritting, and D. T. Denhardt, unpublished data.
RESULTS

ras-transformed 3T3 lines were generated from both wild type and OPN-deficient mice: these cell lines have been previously described (8), and shown to give rise to tumors following subcutaneous injection into syngeneic wild type hosts. These tumors form much more slowly when the injected cells are deficient for OPN production, illustrating the important role that OPN plays in the process of tumorigenesis (8). In other experiments (data not shown), these same cell lines were injected into either wild type or OPN-deficient mice. When the tumors reached 2000–3000 mm³, blood was collected and the tumors were excised. The OPN in 100 μl of serum from animals of different genotypes was precipitated with barium citrate (panel A), and tumor extracts were prepared as described under “Materials and Methods” (panel B). These protein preparations were separated on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and reacted with antibody OP199. The genotype of the tumor and of the host animal is indicated for each set of lanes. For each condition, duplicate or triplicate samples from independent animals were analyzed, except for the −/− tumor in the −/− mouse (panel B, lane 7), where no OPN was detected. In part A, lanes 9–11 are from a different gel, the sample in lane 9 is the same as that in lane 2 for comparison purposes. S, OPN standard: 10 μl of serum-free conditioned medium from wild type ras-transformed 3T3 cells. N, normal serum from a wild type mouse without a tumor: no OPN was detected in this sample. The band migrating near the bottom of the gel in part A is commonly seen in OPN preparations and is most likely the thrombin-cleaved form of the protein.

FIG. 1. OPN protein in serum from tumor bearing mice, and in the tumors themselves. Wild type and OPN-deficient ras-transformed 3T3 cells were injected into either wild type or OPN-deficient mice. When the tumors reached 2000–3000 mm³, blood was collected and the tumors were excised. The OPN in 100 μl of serum from animals of different genotypes was precipitated with barium citrate (panel A), and tumor extracts were prepared as described under “Materials and Methods” (panel B). These protein preparations were separated on 12% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and reacted with antibody OP199. The genotype of the tumor and of the host animal is indicated for each set of lanes. For each condition, duplicate or triplicate samples from independent animals were analyzed, except for the −/− tumor in the −/− mouse (panel B, lane 7), where no OPN was detected. In part A, lanes 9–11 are from a different gel, the sample in lane 9 is the same as that in lane 2 for comparison purposes. S, OPN standard: 10 μl of serum-free conditioned medium from wild type ras-transformed 3T3 cells. N, normal serum from a wild type mouse without a tumor: no OPN was detected in this sample. The band migrating near the bottom of the gel in part A is commonly seen in OPN preparations and is most likely the thrombin-cleaved form of the protein.

FIG. 2. Biochemical fractionation of tumor extracts. Mammary tumors arising in MMTV-c-myc/MMTV-γ-Ha-ras transgenic mice were flash-frozen, powdered, and resuspended in buffer containing 0.2% Triton X-100, and sonicated. The supernatant was retained (soluble lanes) and the pellet was resuspended in the same buffer, and resonicated twice more. After the third wash, the pellet was resuspended in 6 M urea (pellet lanes). Western blotting of these preparations was performed using antibodies for OPN (30 μg of protein/lane, antibody LF123), fibronectin (FN), or laminin (LAM, both 20 μg of protein/lane). +/+ tumor from a wild type mouse; −/−, tumor from an OPN −/− mouse. The position of OPN is indicated on the right. Open arrow, nonspecific proteins cross-reacting with the anti-OPN antibody. St, standards: OPN prep from conditioned medium as in Fig. 1 (OPN panel); 500 ng of bovine plasma fibronectin (FN panel), or 500 ng of mouse laminin (LAM panel).
confirming that these pellets indeed contained the extracellular matrix material. OPN, on the other hand, was localized exclusively in the soluble fraction: no OPN immunoreactivity could be detected in the pellet, at least in the amount of protein loaded in the well of the PAGE gel (30 μg). Cross-reacting proteins identified in both the soluble and pellet fractions are present in both WT and OPN−/− tumors, indicating that they are not OPN. Laminin and fibronectin reactivity appear also in the soluble fraction: this reactivity may be due to soluble protein present in the tumor, or to partial solubilization of the ECM by the Triton/sonication protocol used. Even if the latter is the case, there is a clear differentiation between the pattern of association of OPN and the former proteins with the insoluble pellet.

The localization of osteopontin in tumor tissue was further examined in these spontaneously arising tumors by immunohistochemistry. For these experiments we chose to use the spontaneously arising tumors rather than those resulting from subcutaneous injection of transformed cells, reasoning that the spontaneous tumors would have a more extensive and well-developed extracellular matrix. Accumulation of OPN was variable in these tumors, with areas of high expression and regions where OPN was undetectable (Fig. 3: panel B). Some tumor samples expressed very low levels of OPN (data not shown). In regions where OPN expression was high, however, immunoreactivity appeared over tumor cell cytoplasm. The extracellular matrix in these tumors was identified by staining of adjacent sections for laminin, which was associated particularly with connective tissues and with the basement membrane of blood vessels (Fig. 3: panels A and D, LAM), with some reactivity appearing associated with the tumor cells themselves. Double immunofluorescence for OPN and laminin, examined at higher magnification in a single optical section by confocal microscopy (Fig. 3, panel C), indicated that this laminin reactivity was localized over the cytoplasm of the cells. Thus OPN and laminin co-localize in the cytoplasm of the tumor cells, as these cells are presumably synthesizing both proteins. Laminin was not detected surrounding individual tumor cells: thus, we could not identify the ECM, if any, associated with the tumor cells themselves. However, laminin was clearly localized underlying the tumor vasculature and in regions of connective tissue, and OPN was conspicuously absent from these regions. The arrow in the upper left of Fig. 3C, indicates a cell staining strongly positive for OPN. This cell is directly adjacent to the basement membrane of a large blood vessel, and OPN is clearly not present in this basement membrane. Thus the OPN secreted from this cell does not associate with the nearby basement membrane. OPN expression was not detected in macrophages, identified by F4-80 staining (data not shown). This is in accordance with previous results from in situ hybridization, indicating that OPN in these tumors is produced primarily by the tumor cells themselves (3), and contrasts with results obtained with the subcutaneous tumors (Fig. 1), highlighting the variability of the host anti-tumor response.

"ras"-transformed 3T3 cells in culture generate considerable amounts of OPN in the conditioned medium: we have estimated that the level of OPN in medium conditioned overnight by such cells at confluence can reach 10 μg/ml (data not shown). These transformed cells grow rapidly and are not growth arrested at confluence, so they do not generate substantial extracellular matrix of their own. To determine whether the OPN made by these cells in culture associates with the ECM, wild type and OPN−/− transformed cells were plated onto matrigel and collagen I as surrogate extracellular matri-

![Fig. 3. OPN and laminin localization in mammary tumors.](image-url)
Confluent cells on the different matrices were then fractionated into soluble and ECM components, and the presence of OPN in the different fractions determined by Western blotting. This fractionation was performed using either Triton X-100 (Fig. 4) or EDTA (Fig. 5) to remove the cells. When the cells were plated on either matrigel or collagen and then removed with Triton X-100, only a very slight OPN reactivity could be detected in the ECM fraction: this reactivity is seen in both the WT and OPN −/− samples, and is probably a cross-reacting protein (Fig. 4, A and B). The presence of ECM components exclusively in the ECM fraction was confirmed by reacting parallel blots with antibody to laminin (Fig. 4C). When the cells were plated on matrigel, and then removed with EDTA, again, OPN was undetectable in the ECM fraction, but was observed in the cell lysate, and was abundant in the conditioned medium (Fig. 5A and data not shown). Both laminin and fibronectin, on the other hand, were retained through the multiple washings included in the ELISA procedure. No detectable OPN was retained on wells coated with fibronectin, matrigel, collagen I, or gelatin (Fig. 6A); while slight immunoreactivity was observed in some wells (e.g. matrigel) it was the same whether the wells were incubated with WT or OPN −/− conditioned medium, or with BSA only (Fig. 6A, compare matrigel WT and OPN −/− with matrigel only). OPN in the conditioned medium diluted 1:90 could be readily detected when coated directly on the plastic, either with or without fixation (Fig. 6B). Thus, if any of the OPN from this conditioned medium associates with these matrix components, it is less than 1% of protein present in the conditioned medium.

HUVECs express high levels of the α5β3 integrin (39): function of this integrin is critical for endothelial cell growth and survival in vivo (22). These cells adhere to immobilized OPN with a similar dose response as to other adhesive ligands such as fibronectin and laminin (Fig. 7). Post-translational modification of OPN (phosphorylation and glycosylation) is not required for the protein to support adhesion of these cells (compare mOPN and GST-OPN). HUVECs require adhesion to the extracellular matrix to proliferate, and rapidly undergo apoptosis when held in suspension in the absence of growth factors (40, 41). As tumor OPN appears to be primarily soluble, we have asked whether soluble OPN can have any of the same effects on HUVECs as immobilized OPN. HUVECs were starved for growth factors overnight, and plated on wells coated with fibronectin, or with different concentrations of OPN. In other wells, the cells were prevented from adhering to the dish by plating on BSA. Soluble OPN at different concentrations was added to the cells kept in suspension. These cells were then incubated overnight in the presence of 10 ng/ml aFGF, and their ability to transit the cell cycle assessed by incubation with [3H]thymidine. Thymidine incorporation was similar when the cells were plated on FN, or on all concentrations of OPN tested (Fig. 8A). However, cells held in suspension by plating on BSA were unable to incorporate thymidine, and soluble OPN (sOPN) had no effect on these cells ability to enter the cell cycle.

When HUVECs were plated as described above and incubated overnight in the absence of growth factor, cells held in
suspension (plated on BSA-coated wells) underwent apoptosis. While immobilized OPN at different concentrations or immobilized fibronectin could suppress this apoptotic response, soluble OPN had no effect on the apoptotic response of cells held in suspension (Fig. 8B). Control experiments indicated that GST-OPN coated onto the wells remained immobilized throughout the course of the experiment. Conversely, we have not formally eliminated the possibility that some small fraction of the soluble OPN may become immobilized during the experiment. However, the lack of any effect of soluble OPN above that of BSA alone or GST (Figs. 8, A and B, compare sOPN, BSA, and GST samples) supports the idea that the soluble OPN added to the wells remains soluble throughout the experiment. If there is some immobilization of the soluble OPN, then it is an insufficient amount to have an effect in these assays. Thus, we conclude that while immobilized OPN can support endothelial cell proliferation, and suppress the apoptosis occurring in the absence of adhesion, soluble OPN, such as that associated with tumors, has no such effects. Thus, endothelial cell proliferation and survival is probably not regulated by OPN in tumorigenesis, and the protein is likely acting as a cytokine in either an autocrine or paracrine manner. The target cells of this soluble OPN produced by tumors is still unknown: it could be the tumor cells themselves or a variety of different host cell types.

Osteopontins association with bone matrix as well as its homology (chiefly the RGD sequence) with extracellular matrix proteins has earned it a reputation as an extracellular matrix protein. Structurally, however, the protein has significant differences from well characterized matrix proteins such as fibronectin, laminin, and collagen. OPN is a small protein (~300 amino acids) with no recognized domain or repeat structure (42). Unlike these other matrix proteins, OPN is not known to form organized multimeric structures. Thus, if it were to associate with the insoluble extracellular matrix, it must do so through interaction with other matrix components. In bone, OPN associates with the matrix largely through its high affinity for hydroxyapatite: OPN is not released from bone by chaotropic reagents alone, but requires high concentrations of a calcium chelator such as EDTA for release from the bone matrix (26).

There are numerous reports, however, that OPN binds specifically to components of the extracellular matrix, and that it is a substrate for transglutaminase cross-linking (43, 44). In bone extracts, for example, OPN immunoreactive protein migrates at high molecular weights (44), and is thought to be cross-linked by transglutaminase through conserved glutamine residues. OPN can also be cross-linked in vitro to fibronectin (45). This cross-linking of OPN could serve to immobilize the protein in the extracellular matrix. In addition, OPN in vitro can bind specifically to collagen (46, 47). These observations are taken as evidence for OPNs association with the extracellular matrix in non-mineralized tissues. However, it seems that this latter interaction may not occur readily in soft tissues such as tumors in vivo. In our experiments, binding of OPN to collagen was undetectable in cells in culture: OPN produced by tumor cells failed to associate with collagen coated on the culture dish in either the presence or absence of the cells. Thus, while this association may occur in defined conditions, with purified components, it does not appear to occur at relevant levels in the more complex conditions of the cell culture system used or in tumor tissue. For instance, if OPN were cross-linked in tumor ECM to fibronectin, we would expect to see retention of OPN in the tumor insoluble material, which retains abundant fibronectin (Fig. 2), but this is not the case. No OPN could be detected in the pellet fraction in this experiment.

We have utilized a variety of techniques to isolate extracellular matrix, and see no association of OPN with these different
thymidine was added to 2
incubated for 24 h in the presence of aFGF and heparin. For the last 5 h,

suspension by plating on wells coated with 2% BSA. Cells held in
previously coated with GST-OPN at 1
50

HUVECs were grown to 80%

basement membrane elaborated
extracellular matrix. This is especially clear in regard to the
tumor tissue, OPN does not associate significantly with the
is no evidence of OPN in this preparation.
remove the cells, and likely represents fragments of ECM that
5). This is probably due to the extensive mixing required to
mimic-stimulated pathways (52, 53). Thus it is not surprising
OPN, which binds to both integrin and non-integrin ad-
hesive receptors, can support cell proliferation and survival
when immobilized on a plastic surface (23). However, our
results indicate that in vivo, OPN is unlikely to serve these
functions in the absence of its immobilization in the extracel-
lar matrix. Thus, at least in the case of OPN produced by
tumors (data not shown). The Western blot analysis of Fig. 1
indicates that the production of OPN exclusively by these host
cells can result in levels of OPN in the tumor that approaches
that seen when the tumor expresses OPN. This observation
highlights the extent of involvement of OPN in the host re-
response to tumorigenesis.
OPN has been characterized as a cell attachment molecule in
a variety of models, and this attachment can be both RGD-de-
pendent and -independent (10, 15, 51). Cell attachment is crit-
cal for growth and survival of a wide variety of cell types, in
many cases due to a requirement for signaling through integ-

FIG. 8. Effect of soluble (sOPN) and immobilized OPN on HU-
VEC proliferation and apoptosis. HUVECs were grown to 80%
confluence and made quiescent by overnight incubation in serum-free
medium without growth factors. The cells were then plated on wells
coated with GST-OPN at 1–50 μg/ml (1–50, OPN), fibronectin
(10 μg/ml, FN), with GST protein (10 μg/ml, GST) or held in
suspension by plating on wells coated with 2% BSA. Cells held in
suspension were treated with soluble GST-OPN at different concentra-
tions (sOPN 1–50). Panel A. [3H]thymidine incorporation. Cells were incubated for 24 h in the presence of aFGF and heparin. For the last 5 h,
[3H]thymidine was added to 2 μCi/ml. The cells were then washed, fixed
with trichloroacetic acid, and solubilized prior to counting. Total counts/
well are shown for each substrate or addition. Panel B, apoptosis. Cells
were incubated overnight in the absence of growth factors, plated on
different substrates, and treated with soluble GST-OPN as indicated
above. After 22 h, the cells were labeled with Hoescht 33342 for 15 min.
Floating and adherent cells were collected, fixed with 4% paraformal-
dehyde, and the number of fragmented and intact nuclei determined by
fluorescence microscopy. Percent apoptotic cells is equal to the percent-
age of cells with fragmented nuclei.

matrix preparations. Triton X-100 extraction of cellular com-
ponents yields an ECM preparation that retains diverse pro-
teins such as tenascin (48), apolipoprotein E (36), and plasmin-
gen activator inhibitor (37), but not OPN (Fig. 4). In culture,
removal of the cells with EDTA removes some of the matrix,
as shown by the presence of laminin in the cell lysate fraction (Fig.
5). This is probably due to the extensive mixing required to
remove the cells, and likely represents fragments of ECM that
become detached during this process. However, considerable
laminin and fibronectin remain in the ECM fraction, and there
is no evidence of OPN in this preparation.
The experiments described here indicate that at least in
tumor tissue, OPN does not associate significantly with the
extracellular matrix. This is especially clear in regard to the
basement membrane elaborated in vivo by endothelial cells:
these structures are devoid of OPN immunoreactivity by im-
munohistochemistry and immunofluorescence. Thus, it is un-
likely that OPN facilitates tumorigenesis through a prolifera-
tive or protective effect on endothelial cells, since we also
showed that soluble OPN does not mediate these effects. An
important aspect of the experiments presented here is the
inclusion of the OPN in vivo tumors as controls for the immuno-

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