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Short Communication

Decreased Expression of Ras GTPase Activating Protein in Human Trophoblastic Tumors

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The normally developing placenta undergoes extensive but regulated noninvasive cellular proliferation. Various proto-oncogenes and growth factors have been associated with the regulation of trophoblastic placental growth. Activation of some oncogenes and altered expression of growth factors have been demonstrated in trophoblastic tumors (hydatidiform mole and choriocarcinoma). The ras proto-oncogene plays a key role in the signal transduction cascade of activated growth factors, and is known to be activated or overexpressed in multiple tumor types. Ras GTPase activating protein (RasGAP), a major down-regulator of ras activity, is present at high levels in placenta. To assess the role that RasGAP plays in the development of trophoblastic tumors, we performed immunohistochemical analyses with anti RasGAP antibodies of normal placentas, hydatidiform moles, invasive moles, and malignant choriocarcinomas. Normal placentas and noninvasive hydatidiform mole displayed intense positive staining confined to trophoblasts, whereas no staining was observed in the trophoblasts of invasive moles or choriocarcinomas. Thus, there was an inverse correlation between expression levels of RasGAP protein and the invasive potential and malignant phenotype in human trophoblastic tumors. The data indicate that RasGAP may play a regulatory role in trophoblast proliferation and that abolishing its activity may be associated with malignant transformation of these cells. (Am J Pathol 1995, 146:1073–1078)

Extensive dysregulated cellular proliferation and an ability to invade normal surrounding tissues with resultant metastases are the hallmarks of malignant tumor development. A similar degree of trophoblastic proliferation is observed in the placenta, yet local invasion or metastases never occur in normally developing placenta. In addition, the placenta may be a barrier that protects maternal tissues from being invaded by the antigenetically different fetus. It is therefore particularly important to elucidate the molecular basis of the mechanisms underlying the unique placental properties, as it may lead to a better understanding of the processes of tumorigenesis and metastasis. Insight into these mechanisms may be gained by analyzing abnormal placentas. Complete hydatidiform mole, a benign condition caused by fertilization and activation of an oocyte lacking the maternal genome,[1] is characterized by uncontrolled trophoblastic proliferation and, at times, local invasion into the myometrium (invasive mole).[2–4] Approximately 3% of patients with complete hydatidiform mole (ie, moles with two sets of paternal genes and no maternal genes) develop choriocarcinoma, a highly aggressive trophoblastic tumor.[2–4] Thus, pla-

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centa, hydatidiform mole, and choriocarcinoma represent a spectrum of trophoblastic cell proliferation, with normal trophoblasts at one end and the highly malignant choriocarcinoma cells at the other end. The exact molecular mechanisms involved in the initiation and progression of benign and malignant trophoblastic tumors are largely unknown. Activation of several oncogenes, including *myc* and *fms*[5] and *mos*, *src*, *abl*, *erb*-A, and *ras*,[6,7] has been demonstrated in hydatidiform mole and choriocarcinoma and hence implicated in the tumor development process.

The *ras* gene family encodes membrane-associated, guanine nucleotide-binding proteins (p21) that play a pivotal role in the signal transduction cascade that links growth factors via their respective receptors with intracellular events culminating in cellular proliferation and differentiation.[8] The Ras proteins cycle between an active guanosine triphosphate (GTP)-bound form and an inactive, guanosine diphosphate (GDP)-bound form.[9] The weak intrinsic GTPase activity of Ras proteins is greatly enhanced by the actions of GTPase activating proteins (GAPs), such as p120 RasGAP[10] and neurofibromin, the product of the neurofibromatosis type I (NF1) gene.[11] Biochemically, nearly all of the activating point mutations detected in the *ras* genes in a wide variety of human tumors[8] decrease the intrinsic GTPase activity of Ras[12] and render it insensitive to stimulation by GAPs.[13] These observations lead to the hypothesis that at least some of the transforming activity of mutant *ras* is conferred by the Ras protein being constitutively activated in its GTP-bound state. Thus, theoretically,[4] ablating the ability of GAPs to effectively down-regulate activated Ras, could result in a similar tumorous phenotype. Such an ablation could result from either an underexpression of a nonmutated protein or from an inactivating mutation(s) in the catalytic domain (ie, the region of GAP that is responsible for the down-regulation of Ras). Indeed, both mechanisms have been previously demonstrated for neurofibromin.[14,15]

We report here that immunohistochemically detectable RasGAP protein is expressed at very high levels in trophoblasts of normally developing placenta and that the transition from benign mole to malignant choriocarcinoma seems to correlate with markedly reduced levels of RasGAP protein.

Materials and Methods

Patients and Tissues

Formalin-fixed, paraffin-embedded specimens of three normal term placentas, one 12-week-gestation placenta, three complete hydatidiform moles, two invasive moles, and two choriocarcinomas were obtained from the Department of Pathology, Karolinska Hospital, Stockholm, Sweden. Three additional samples of choriocarcinoma were obtained from the Department of Pathology, Hospital Felicio Rocho, in Belo Horizonte, Brazil. Hydatidiform mole and choriocarcinoma were diagnosed by conventional criteria[2,16,17] as determined by two independent pathologists.

Immunohistochemical Analysis

Deparaffinized, rehydrated sections of all tissues were digested with 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37 C, rinsed in phosphate-buffered saline, and treated with 0.3% H_2O_2 in methanol for 30 minutes at room temperature to quench endogenous peroxidase activity. The sections were then stained according to the indirect peroxidase method.[18] Briefly, the sections were incubated with 2% normal rabbit serum for 15 minutes at room temperature and subsequently with sheep polyclonal anti-GAP antibody raised against a GAP synthetic peptide (OP-11–3517, Cambridge Research Biochemicals, Cheshire, UK), diluted 1:100 for 2 hours at room temperature. The sections were then incubated with peroxidase-conjugated rabbit anti-sheep antibody diluted 1:30 in 10% human serum. For detection, 0.2 mg/ml 3-amino-9-ethylcarbazole was used. Control sections consisted of serial sections from the same blocks that were similarly processed and analyzed during the experiment, except that no anti-GAP antibody was used. To ascertain the specificity of the staining, immunoadsorption experiments were performed (see below).

All tissues were additionally immunostained with rabbit GAP polyclonal antiserum raised against the holoprotein (the generous gift of Dr. Frank McCormick and George Martin, Onyx Pharmaceuticals, Richmond, CA),[19] with an avidin-biotin-peroxidase complex system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. The rabbit GAP polyclonal antiserum was diluted 1:1000 from the original serum volume and incubated with sections for 2 hours at room temperature. Control sections were incubated with normal rabbit serum diluted to the same antibody concentration as the primary antibody.

For both antibodies, sections were counterstained with Harris hematoxylin. Both antibodies recognize both types of GAP,[19,20] which are the result of alternative splicing of the same gene and cannot be used

to distinguish between them, and have been shown to be specific and sensitive by Western blot analysis.[19,20]

Each slide was independently examined by two experienced pathologists. The intensity of immunostaining was graded as follows: −, no observed immunostaining; +, weak but definite staining; ++, stronger positive staining and +++, intense staining.

Immunoadsorption

To ascertain the specificity of immunostaining, we performed immunoadsorption with sheep polyclonal antibody to GAP (Cambridge Research Biochemicals) raised against the GAP synthetic peptide (OP-11–3517, Cambridge Research Biochemicals). Immunoadsorption was done following the recommendation of the manufacturer. Briefly, 20 µl of the GAP peptide was added to 1 ml of a 1:100 dilution of sheep anti-GAP antibody. The mixture was incubated for 3 hours at room temperature, and immunohistochemical analysis was performed according to the indirect peroxidase method.[18] Final concentrations of peptide in the reaction were 10 µg/ml, 10 ng/ml, and 0.01 ng/ml. Control slides were processed at the same time by the same protocol, except that no peptide was added to adsorb the antibody.

Results

All four samples of normal early and late gestation placenta showed intense immunostaining for GAP protein in villous syncytiotrophoblasts (Figure 1a, b). The immunostaining of the intermediate trophoblasts in the decidua and cellular columns was positive, though less intense (++) (Figure 1a). We were not able to assess whether immunoreactivity differed between cyto- and syncytiotrophoblasts in these samples. Immunostaining decreased in a dose-dependent manner as the concentrations of the GAP peptide used to adsorb was increased; a 10 µg/ml peptide concentration completely abolished staining in syncytiotrophoblasts (Figure 2). Occasional immunopositive mast cells remained positive even after immunoadsorption, indicating false positive staining in these cells. Identical staining patterns were observed when rabbit anti-GAP antibody was used (data not shown).

Samples of three different noninvasive benign hydatidiform moles also showed positive immunostaining in trophoblasts (Figure 1c). The intensity of the staining was somewhat weaker then the normal placenta staining (scored as ++ in our scoring system in some fields and +++ in others), but detectable immunostaining was consistently seen in all samples. However, in two invasive moles and four of the five choriocarcinoma tissues, there was no immunostaining in cyto- and syncytiotrophoblasts (scored as −), indicating reduced or absent expression of GAP protein (Figure 1d). In one choriocarcinoma, occasional neoplastic syncytiotrophoblasts were weakly positive (+), but the cytotrophoblasts were consistently negative (Figure 1e, f). None of the negative controls exhibited any immunostaining with GAP antibody. Results were similar for both types of antibodies (rabbit and sheep).

Discussion

In this study, RasGAP protein was underexpressed in benign and malignant human trophoblastic tumors. Furthermore, there was an inverse correlation between these reduced RasGAP levels and malignant phenotype and invasive potential of these tumors. Intermediate trophoblasts, locally invasive but benign cells that are strictly regulated, express abundant RasGAP, and unregulated invasive choriocarcinomas expressed almost no RasGAP protein as detected by immunohistochemical analysis. Likewise, a previous study with immunoblotting of RasGAP protein in two human choriocarcinoma cell lines demonstrated that RasGAP levels are significantly decreased (up to 1000-fold) as compared with normal placenta.[21]

It has been suggested that proto-oncogenes have a role in regulating and coordinating the massive proliferation and differentiation of many cell types observed during normal placental development.[22–25] Similarly, mutated proto-oncogenes (activated oncogenes) have been implicated in the tumorigenesis of trophoblastic tumors.[5–7] In addition, the expression of several growth factors has been found to be altered at various stages of placental development[25–27] as well as in abnormally proliferating placental tumors, ie, hydatidiform moles and choriocarcinomas.[25,27–29] Notably, overexpression of platelet-derived growth factor β (PDGFβ) and PDGFβ receptor upon malignant transition of trophoblasts has also been demonstrated.[29] Acting via its specific receptor, PDGFβ, a potent *in vitro* mitogen, sets in motion a cascade of intracellular phosphorylation events that culminate in cellular proliferation.[30,31] One of the major intracellular pathways downstream of PDGF involves the activation of the Ras protein.[8,31] Taken together, these observations suggest that an abnormal signal transduction cascade involving growth factor receptor, Ras protein, and GAP play a role in trophoblastic cell proliferation.

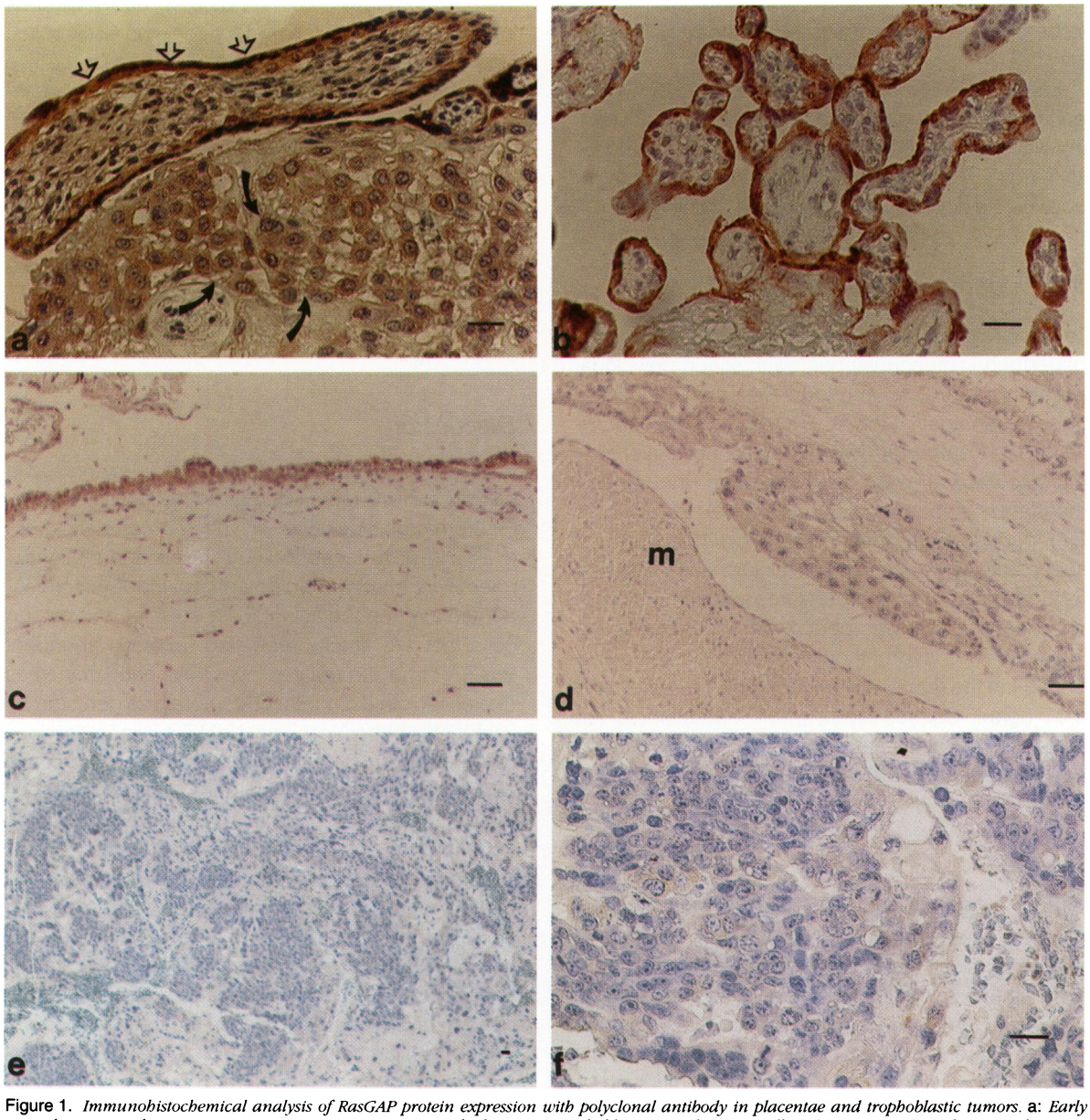

Figure 1. *Immunohistochemical analysis of RasGAP protein expression with polyclonal antibody in placentae and trophoblastic tumors.* **a:** *Early second trimester placenta. Note intense immunoreactivity in both syncytiotrophoblasts in a chorionic villous (open arrows) and intermediate trophoblasts in a cellular column (arrows).* **b:** *Term placenta displaying intense immunoreactivity in syncytiotrophoblasts.* **c:** *Noninvasive complete mole with moderate immunoreactivity confined to the trophoblastic cell layer.* **d:** *Invasive mole without detectable immunoreactivity by trophoblasts m, myometrium.* **e** *and* **f:** *Choriocarcinomas. Note lack of staining in cytotrophoblastic cells. Very weak immunoreactivity was seen in occasional syncytiotrophoblasts. Scale bars represent 25 μ.*

In this hypothetical model, the normal trophoblast can attain a proliferative advantage by either overexpressing the ligand and its receptor (ie, PDGFβ and its respective receptor) and thus perpetually be activated and driven to proliferation in an autocrine fashion. Alternatively, underexpression of GAP, one of the major down-regulators of an activated Ras protein, which lies downstream of the PDGF receptor, could also result in an unregulated trophoblast proliferation. In this model, GAP acts as a tumor suppressor: when GAP is expressed at normal levels, there is no abnormal cellular growth. Evidence for the ability of GAP to act as a tumor suppressor comes from *in vitro* studies, showing that overexpression of GAP may prevent the *ras*-induced[32] and *src*-induced[33] transformation of rat fibroblasts. Another protein that substantially contributes to the down-regulation of activated *ras* is neurofibromin, the product of the neurofibromatosis type 1 (NF1) gene.[11] Neurofibromin shares sequence and functional homologies with RasGAP, and its levels have been shown to be markedly reduced in conjunction with ras being in its active, GTP bound state

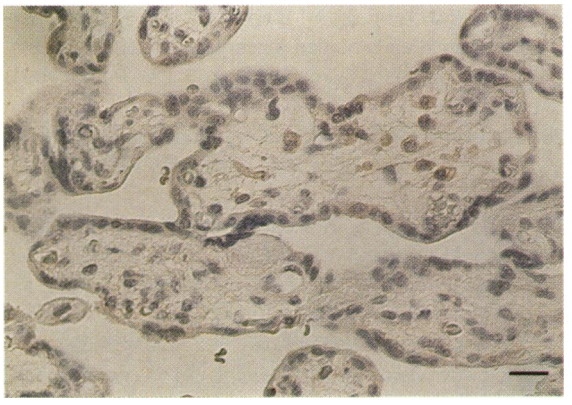

Figure 2. *Immunoadsorption with 10 mg/ml RasGAP peptide before immunostaining of normal placenta (same sample as Figure 1b) with GAP antibody. There is no immuno-staining. Scale bars represent 25 μ.*

in malignant Schwannoma cell lines.[14] Additional proof of the tumor suppressor activity of RasGAP comes from the reversion of tumorous phenotype of these Schwannoma cells upon transfection and expression of full length RasGAP.[14]

One of the hallmarks of a tumor suppressor gene is allelic loss in tumorous tissue when compared with normal, nontumorous tissue. This mechanism inactivates one copy of the gene, and the other allele is presumably inactivated by a minor genetic abnormality (eg, a point mutation or minor deletion).[34,35] To the best of our knowledge, no studies were systematically performed in trophoblastic tumors to assess the existence and extent of allelic losses. Moreover, in the few studies in which choriocarcinoma and moles were cytogenetically analyzed, there were no abnormalities of chromosome 5, where the GAP gene is located. Furthermore, the complete hydatidiform mole totally lacks the maternal genome and hence is homozygous, thus making the search for allelic losses in these tumors pointless. Microcell fusion experiments also support the involvement of a tumor suppressor gene in choriocarcinoma tumorigenesis. Using this technique to introduce individual chromosomes derived from normal cells into a choriocarcinoma cell line, Miyamoto and co-workers[34] reported that a gene on chromosome 7 may exert tumor suppressor activity in choriocarcinoma.

Another possible mechanism to inactivate the remaining copy of a tumor suppressor gene is by hypermethylation of its regulatory elements, which results in underexpression of the gene. Hydatidiform moles and choriocarcinomas are known to have imprinted genes, which is tightly associated with hypermethylation.[37,38] Beckwith-Weidemann syndrome is an example of a disease in which abnormal organ development and a predisposition to Wilms' tumor formation is also associated with parental allele-specific expression of imprinted genes located to 11p15.[39]

In summary, the apparent underexpression of RasGAP protein in invasive hydatidiform moles and choriocarcinomas and the inverse correlation with malignant phenotype and invasive potential suggest that RasGAP plays an important role in human trophoblastic tumors formation.

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