Nuclear Injection of Anti-pigpen Antibodies Inhibits Endothelial Cell Division*

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Endothelial cell proliferation is required for angiogenesis in both embryonic and adult tissues. In rat brain tumors, it has recently been shown that the nuclear protein pigpen is expressed selectively in endothelial cells of developing microvasculature but not in the established peritumoral vessels (Blank, M., Weinschenk, T., Priemer, M., and Schluesener, H. (2001) J. Biol. Chem. 276, 16464–16468). This finding suggests that pigpen may be important for promoting the undifferentiated, or “angiogenic” endothelial cell phenotype. Our studies show that pigpen protein and mRNA are expressed in actively dividing endothelial cells and down-regulated as they become confluent. Protein distribution is regulated in a cell cycle-dependent manner. We conclude that this expression pattern is important for and not simply ancillary to proliferation because nuclear microinjection of anti-pigpen Fab fragments inhibited endothelial cell division. Moreover, expression of the proliferating cell marker Ki67 was inhibited in antibody-injected cells. The absence of Ki67 suggests exit from rather than arrest within (for example, at the G1/S interface) the cell cycle. Together with earlier observations on the structure and expression of this molecule, our data support the hypothesis that pigpen helps regulate endothelial cell differentiation state.

Angiogenesis is the development of new blood vessels from existing vasculature. It begins early and continues throughout embryonic development, extending early rudiments of the vascular system to meet the nutritive requirements of growing tissues. In adult tissues angiogenesis is a relatively rare and tightly regulated event. Endothelial cells (EC)¹ must therefore be transformed from a quiescent, non-proliferative state to an undifferentiated/proliferative phenotype that provides the building blocks and framework for new vessels. This process occurs normally during wound healing and cyclic reproductive events but can also be triggered by a variety of pathological conditions resulting in considerable tissue damage (1). New blood vessel development is critical for tumor growth and metastasis, for example. In the eye, angiogenesis is involved in more than 20 disorders (2), including neovascular glaucoma, age-related macular degeneration, and proliferative diabetic retinopathy.

Great progress has been made in our understanding of extracellular factors governing angiogenesis. Control of EC phenotype is generally thought to be regulated by a balance of stimulators and inhibitors. We have a great deal more to learn about the downstream effectors of angiogenesis, however, especially the nuclear factors that convey incoming signals into the new genetic program. Induction of several well known nuclear regulatory molecules has been shown to accompany angiogenic stimulation. For example, analogues of the immediate early genes, c-fos, c-jun, and egr-1 are induced in phorbol 12-myristate 13-acetate-stimulated EC (3). Phorbol 12-myristate 13-acetate also induces EDG-2, a human homologue of the Xenopus G10 protein. Induction of the DNA-binding homeobox protein Hox D3 has been shown not only to accompany but to trigger expression of the angiogenic phenotype in avian EC (4). Still there are many gaps to be filled. It is certain that other characterized regulatory proteins will be fitted into this scheme, and equally likely that new ones will be discovered.

There is evidence to suggest that the nuclear protein pigpen functions in the proliferative phase of angiogenesis. This molecule displays a transcription activation domain as well as consensus motifs associated with RNA binding (5). In the latter case, we have evidence to suggest that pigpen interacts selectively with mRNAs, notably those of positive cell cycle regulators such as Bub1, E2F4, and katanin (6). Importantly, it was recently shown that pigpen is highly expressed in rat brain tumor microvessels but is absent in mature brain vasculature, even in zones immediately adjacent to the tumor (7). Together with other observations on the up-regulation of pigpen in wound vascular cell monolayers (5, 7, 8), these data suggest that pigpen’s expression pattern underlies a functional role in regulating EC phenotype.

We tested the hypothesis that pigpen is important for cell division by microinjection of anti-pigpen Fab fragments into the nuclei of EC in proliferating cultures. More than 200 individual cells were injected, tracked, and assayed for progression through mitosis. We found that cell division was inhibited by ~50%. Anti-pigpen arrested cells appeared to be thrown out of the cell cycle as judged by the absence of the ubiquitous cycling cell marker Ki67. Our results suggest that pigpen expression is not only coincident with proliferation in EC but is important for maintaining the proliferative/undifferentiated phenotype required for the early stages of angiogenesis.

EXPERIMENTAL PROCEDURES

Source and Culture of Cells—Fetal bovine nortic EC used in this study were isolated according to the methods of Fenselau and Mello (9) and have been characterized and described previously (10). Cells were cultured on sterile glass coverslips in minimal essential medium containing 15% fetal bovine serum plus 100 units/ml penicillin and 100 μg/ml streptomycin. In some experiments, cells were arrested in G₁, G₂/M, or S phase by the addition of the calcium ionophore A23187 (20 μM) or the diuretic bumetanide; CB, coiled bodies.

The abbreviations used are: EC, endothelial cell(s); AB, amiloride + bumetanide; CB, coiled bodies.

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¹ The abbreviations used are: EC, endothelial cell(s); AB, amiloride + bumetanide; CB, coiled bodies.
Red-conjugated dextrans, 10 mM NaH₂PO₄, and 70 mM KCl, pH 7.2. Keefe O’topascals, respectively. The microinjection buffer has been described by
tion system with duration and pressure settings of 0.5 s and 30 hec-
slips were microinjected using an Eppendorf automated microinjec-

The IgG concentration in microinjection buffer was 1 mg/ml.

microinjection buffer using Centricon microfiltration devices (Amicon).

of a subsequent protein A affinity column and pressure-dialyzed into

chromatography and reduced to Fab fragments by papain digestion as

slides with Vectashield (Vector Laboratories, Burlingame, CA).

purchased from Sigma. Coverslips were mounted on glass

solution. Monoclonal anti-Ki67, purchased from Immunotech

min in primary antibody. Anti-pigpen was diluted at 1/500 in blocking

30-min incubation in blocking solution (1.5% goat serum in PBS) and 60

Following microinjection cells are washed two times in minimum es-

for immunochemistry. Culture medium was

ing with a combination of amiloride + bumetanide (AB) as described by
Panet et al. (11). Briefly, confluent cells were harvested by trypsiniza-
transfered to nitrocellulose, and probed with anti-pigpen or preim-
mune serum from the same rabbit. The detection system used was a
peroxidase-conjugated secondary antibody with hydrogen peroxide and
1,4-dichloronaphthol substrates. India ink was used to stain total pro-
tein in parallel lanes. The results of Western analysis are shown in Fig.

Immunological—The specificity of polyclonal anti-pigpen was examined
by Western blot analysis using methods described previously (5).
Whole EC lysates were resolved by polyacrylamide gel electrophoresis,
and probed with anti-pigpen or preimmune serum.

For immunohistochemistry of cultured cells, culture medium was
removed with three brief rinses in PBS prior to fixation in 2.5%
parafomaldehyde in PBS (5 min) followed immediately by permeabi-
and post-fixation in 0.5% Triton X-100 (5 min) and 100% methan-
ol (8 min, −20 °C). Three 10-min rinses in PBS were followed by a
30-min incubation in blocking solution (1.5% goat serum in PBS) and 60
min in primary antibody. Anti-pigpen was diluted at 1/500 in blocking
solution. Monoclonal anti-Ki67, purchased from Immunotech
(Marseille, France), is supplied in its own blocking solution and was
used directly from the bottle. Fluorescently labeled secondary antibo-
dies were purchased from Sigma. Coverslips were mounted on glass
slides with Vectashield (Vector Laboratories, Burlingame, CA).

IgGs for microinjection were purified by protein A-Sepharose affinity
chromatography and reduced to Fab fragments by papain digestion as
described by Goding (12). Fab fragments were collected in the effluent
of a subsequent protein A affinity column and pressure-dialyzed into
microinjection buffer using Centricon microfiltration devices (Amicon).
The IgG concentration in microinjection buffer was 1 mg/ml.

Microinjection—Cells cultured on CELLocate (Eppendorf) glass cov-
erslips were microinjected using an Eppendorf automated microinjec-
tion system with duration and pressure settings of 0.5 s and 30 hec-
topascals, respectively. The microinjection buffer has been described by
O’Keefe et al. (13) and contains 15 mg/ml lysine-fixable 70-kDa Texas
Red-conjugated dextrans, 10 mM NaH₂PO₄, and 70 mM KCl, pH 7.2.
Following microinjection cells are washed two times in minimum es-

tential medium + 15% fetal calf serum and cultured under standard
conditions until fixed for subsequent analysis. Pre-immune Fab frag-
ments were injected under identical conditions as a control in all
experiments.

RESULTS

Pigpen Expression Is Regulated—Pigpen is expressed ubiquitously in proliferating EC cultures; however, both the protein
and its mRNA are sharply down-regulated when cells become
confluent (Fig. 2). These observations are consistent with other
reports linking pigpen expression to the undifferentiated phe-

FIG. 1. Selectivity of anti-pigpen in Western blots. Lane A, total
protein, stained with india ink; lane B, probed with anti-pigpen at
1/1000 dilution; lane C, preimmune control at 1/1000 dilution. Each
lane was loaded with 50 μg of whole EC lysate. Positions of kDa
standards indicated on the left. The diffuse band and dots at the bottom
of lane C are dye (pyronin Y) and hand notation, respectively, used to
identify the blot lane.

FIG. 2. Pigpen expression in proliferating and quiescent EC. Shown are sparse (A), subconfluent (B), and confluent (C) EC cultures
labeled with anti-pigpen. D shows Northern analysis of pigpen mRNA
levels in three equivalent cultures, from left to right. Each lane is loaded
with 2 μg of EC poly(A)⁺ RNA. Protein and mRNA expression are
dramatically reduced as cells attain a quiescent phenotype. Expression
is up-regulated rapidly and locally if a wound is made in the cell
monolayers (5,8).
notype both in vitro and in vivo (5, 7, 8). Pigpen is expressed at all stages of the cell cycle, but its distribution is regulated in a cell cycle-dependent manner (Figs. 3 and 4). In G1, there are ~17 pigpen-containing foci (coiled bodies; CB) per cell. This number declines to 10 during progression through S and G2 phases. During this same period nuclear envelope staining appears more prominent. Pigpen foci reach a minimum average of 1 per cell during anaphase and then begin to increase in number again before the completion of M phase. Two observations consistent with past findings were that: 1) pigpen-containing CB can be found outside the nuclear zone during late telophase (and indeed may persist in the cytoplasm, as may be noted in Fig. 2B); 2) throughout M phase pigpen-containing CB seem to associate preferentially with the mediolateral, poleward aspect of chromosomes. It is possible that this localization is passive in some respect, e.g. if CB are excluded from the metaphase figure and remain pressed

\footnotesize{\textsuperscript{2}S. R. Alappat, M. Zhang, M. A. Alliegro, M. C. Alliegro, and C. A. Burdsal, submitted for publication.}

FIG. 3. Pigpen is expressed throughout the endothelial cell cycle; its distribution is cyclically regulated. Cells were double-labeled with anti-pigpen (shown in green) and anti-Ki67 (red), the latter was used to determine cell cycle phase. Overlays of the two images are shown in the right-hand column. A–C, G1 phase; D–F, S/G2; G–I, metaphase; J–L, anaphase; M–O, telophase. A marked change in the number of pigpen-containing foci (coiled bodies) was observed, with a maximum in G1 and a minimum in anaphase (quantitation shown in Fig. 3).

FIG. 4. Cyclic fluctuation in the population of pigpen-containing coiled bodies. Data were derived by random selection of at least 100 Ki67-staged cells in three separate trials (range from 100 to 103 cells). Vertical bars represent standard errors. Data for M phase are divided into prophase (P), metaphase (M), anaphase (A), and telophase (T). All variations from stage to successive stage were determined to be statistically significant (p < 0.05) by analysis of variance.

FIG. 5. Fluorescence redistribution in dividing endothelial cells. Anti-pigpen Fab fragments were injected into the nucleus of 200 EC. The microinjection buffer included labeled dextrans as a marker, and a fluorescence redistribution assay was used to determine the fate of each cell 20 h later. In undivided cells, Texas Red-labeled dextrans remain compartmentalized in the nucleus (A). Nuclear envelope breakdown at the onset of M phase redistributes dextrans throughout the cell (B).
pigpen-induced down-regulation of Ki67 expression. Cells were fixed and prepared for immunofluorescence 20 h after treatment to block proliferation. In A, EC were arrested in G1 with amiloride-bumetanide for comparison and labeled with a monoclonal antibody to the cell cycle marker Ki67. The antigen is expressed in the fine, granular pattern characteristic of cells in G1 (see Fig. 2A, for example). In anti-pigpen arrested EC (B–D), however, Ki67 antigen was not detected. B, nucleus of an injected cell viewed with phase contrast. The grid markings of the CELLocate coverslip visible in this panel are outlined in white in C and D. C is the same cell, showing fluorescence of Texas Red-conjugated dextrans. D, same cell labeled with anti-Ki67.

against the periphery of poleward-moving chromosomes. However, their initial juxtachromosomal positioning in metaphase, when net chromosomal movement is zero or is directed toward the metaphase plate, would appear to discount this possibility and suggest some bona fide interaction.

Pigpen Is Important for Endothelial Cell Proliferation—Given the multifunctional nature of pigpen (including transcription activation and RNA binding domains), its down-regulation in quiescent EC, and its ubiquitous expression in cycling cells, it is a reasonable hypothesis that it functions in maintenance of the proliferating (dedifferentiated) EC phenotype. To test this hypothesis, we examined the effects of nuclear-injected anti-pigpen antibodies on EC proliferation. The microinjection buffer included 70-kDa Texas red-labeled dextrans that both enabled tracking of individual cells on the day following injection and provided a simple means to assay for progression through M phase via fluorescence redistribution. In two experiments, slightly over 200 cells were injected with anti-pigpen Fab fragments or pre-immune Fab fragments as a control. The injected cells were cultured for 20 h to permit at least one round of cell division, then fixed. Divided cells were identified by cytoplasmic fluorescence due to redistribution of dextrans upon nuclear envelope breakdown (Fig. 5). In cells that had not entered M phase, dextrans remained compartmentalized in the nucleus. We found that the difference between control and experimental groups was marked (Fig. 6A); control cultures exhibited almost twice the number of divided cells compared with those injected with anti-pigpen Fab fragments.

It may be that our estimate for inhibition is conservative, depending on whether or not pigpen functions during a discrete phase of the cell cycle. If pigpen were required during G1, for example, then cells injected during S or G2 would likely progress through mitosis and be tallied among the uninhibited. This may be reflected in the relatively high incidence of binucleate cells observed in the experimental group (24 versus 5% for controls; Fig. 6B). One explanation for this finding is that some cells that failed to undergo cytokinesis are added to those blocked prior to karyokinesis, the level of inhibition resulting from injection of anti-pigpen approximates 72%.

Effect of Anti-pigpen Injection on Ki67 Expression—Ki67 is a nuclear protein expressed in proliferating cells but not in non-cycling (G0) cells. Similar to the proliferating cell nuclear antigen and several other well characterized molecules, Ki67 exhibits a repeating pattern of regulated expression and redistribution during the cell cycle (14). It has been used extensively as a marker in human tumor diagnostics (the Ki67 labeling index) and as a reliable cell cycle monitor (see Fig. 3, for example). During G1, Ki67 is present in the nucleoplasm at low levels in a fine, granular pattern. In S and G2 phases Ki67 becomes highly concentrated in nucleoli, and in M phase anti-Ki67 stains chromosomes intensely. EC arrested in G1 with AB exhibit a Ki67 expression pattern consistent with the G1 conformation seen in cycling cells. An example is shown in Fig. 7A...
to be compared with arrest induced by anti-pigpen injection. In the latter cells, Ki67 expression was observed to be greatly attenuated and often completely ablated (Fig. 7, B–D). The lack of Ki67 in these cells suggests that they are not arrested within the cell cycle, as are AB-arrested cells, but have exited the cell cycle and entered a more differentiated state.

We examined the distribution of two other nuclear antigens in injected cells including the nucleolar protein fibrillarin and the spliceosomal protein SC35. In both cases the distribution and apparent expression level appeared indistinguishable from control (pre-immune) or non-injected cells (Fig. 8). These results suggest that the effects of anti-pigpen injection on Ki67 expression and EC proliferation were not likely due to nonspecific or widespread effects on nuclear organization.

**DISCUSSION**

Pigpen’s highly regulated expression pattern both in vitro (5, 8) and in situ (8), coupled with bioactivities and structural elements including a zinc finger, transcription activation, and RNA and carbohydrate binding domains (6), provides compelling circumstantial evidence that it is a regulator of EC phenotype. Additional evidence that this molecule is involved in angiogenesis was recently reported by Blank et al. (7) using an experimental rat brain glioma model. Pigpen was highly expressed in newly forming microvessels within the tumor mass but was notably absent from mature vessels, even in peritumoral zones.

In this report we provide the first experimental evidence to support the hypothesis that pigpen plays a role in angiogenesis via regulation of EC proliferation. We found that pigpen expression is not only restricted to proliferating EC, but cell cycle progression can be substantially perturbed by microinjection of anti-pigpen antibodies in the nucleus of proliferating EC. Without EC proliferation, developing tissues, whether embryonic or pathologic, lack the primary building blocks and scaffolding required for new blood vessel development.

Given the variety of structural motifs the protein exhibits and its subcellular distribution, pigpen could exert its influence at multiple levels from gene activation to RNA processing or transport. Moreover, its expression throughout the cell cycle, marked by a regulated distribution pattern, make it possible for pigpen to play different roles at different times. Identifying and following the biogenesis of gene products downstream from pigpen will enable us to define its function(s).

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