Article

Nucleolin expressed at the cell surface is a marker of endothelial cells in angiogenic blood vessels

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A tumor-homing peptide, F3, selectively binds to endothelial cells in tumor blood vessels and to tumor cells. Here, we show that the cell surface molecule recognized by F3 is nucleolin. Nucleolin specifically bound to an F3 peptide affinity matrix from extracts of cultured breast carcinoma cells. Antibodies and cell surface biotin labeling revealed nucleolin at the surface of actively growing cells, and these cells bound and internalized fluorescein-conjugated F3 peptide, transporting it into the nucleus. In contrast, nucleolin was exclusively nuclear in serum-starved cells, and F3 did not bind to these cells. The binding and subsequent internalization of F3 were blocked by an antinucleolin antibody. Like the F3 peptide, intravenously injected antinucleolin antibodies selectively accumulated in tumor vessels and in angiogenic vessels of implanted "matrigel" plugs. These results show that cell surface nucleolin is a specific marker of angiogenic endothelial cells within the vasculature. It may be a useful target molecule for diagnostic tests and drug delivery applications.

Introduction

Tumor growth is critically dependent on angiogenesis, which is the sprouting of new blood vessels from existing ones (Hanahan and Folkman, 1996). Angiogenic vessels differ from normal vessels in their morphological and molecular characteristics. The molecular markers of angiogenic vessels include endothelial growth factor receptors, integrins, proteolytic enzymes, and extracellular matrix components (Ruoslahti, 2002), as well as membrane proteins of unknown function (St Croix et al., 2000; Christian et al., 2001a). A specific marker for tumor lymphatics has also been described previously (Laakkonen et al., 2002).

The molecular markers that distinguish tumor vasculature from that of normal tissues are important in a number of ways. Many of the molecules that are selectively expressed in tumor blood vessels play a functional role in development and maintenance of new blood vessels. Examples include endothelial cell growth factor receptors and integrins (Eliceiri

and Cheresh, 1999; Ferrara and Alitalo, 1999; Hynes, 2002), matrix metalloproteases (Brooks et al., 1998; Bergers et al., 2000), and aminopeptidase N (Pasqualini et al., 2000). Blocking the function of these proteins inhibits angiogenesis. Furthermore, these and other molecules selectively expressed in tumor vasculature can be made use of in targeting diagnostic and therapeutic agents in tumors (Arap et al., 1998; Nilsson et al., 2001; El-Sheikh et al., 2002; Hood et al., 2002).

The identification of additional tumor blood vessel markers helps in the understanding of angiogenesis and could be useful for tumor targeting. We set out to identify the molecule, "receptor," that is recognized by a tumor-homing peptide recently identified by our laboratory. This peptide, F3, was discovered in a screening procedure that used a phage-displayed cDNA library and combined ex vivo screening on cell suspensions prepared from mouse bone marrow and in vivo screening for tumor homing. F3 is a 34–amino acid fragment of a high mobility group protein, HMG2N (Porkka et al., 2002). F3 homes to the vasculature of various types of tumors by binding to the endothelial cells. F3 also binds to a subpopulation of bone marrow cells that may be precursors for endothelial cells. In some tumors, the F3

Abbreviation used in this paper: HUVEC, human umbilical vein endothelial cell.

peptide also recognizes the tumor cells. A striking property of the F3 peptide is that it is internalized by its specific target cells and transported to the nucleus.

We have now identified cell surface–expressed nucleolin as the receptor for F3 on tumor cells and angiogenic endothelial cells. Cell surface nucleolin expression is a novel angiogenesis marker. It provides a tool for studying tumor angiogenesis, including the contribution of precursor cells to this process, and for targeting drugs into tumors.

Results

Nucleolin binds to F3 in affinity chromatography

The F3 peptide binds to and accumulates within both tumor endothelial cells and tumor cells in vivo (Porkka et al., 2002). Because F3 also binds to cultured tumor cells such as the human breast carcinoma cell line MDA-MB-435, we decided to use this cell line to identify a receptor for F3. Affinity chromatography of MDA-MB-435 cell extracts on an F3 peptide affinity matrix revealed a major F3-binding band at a molecular mass of 110 kD and several bands in the 20-kD range that did not bind to a control peptide matrix (Fig. 1 A). Mass spectrometry analysis indicated that the 110-kD band is nucleolin. Although the calculated mass of nucleolin is 76 kD, it migrates at 110 kD in SDS-PAGE, most likely because of posttranslational modifications and high content of negatively charged amino acids in the NH_2-terminal region of the protein (Harms et al., 2001). The 20-kD range bands were identified as various histones.

The identification of the 110-kD protein as nucleolin was confirmed by immunoblotting. A monoclonal antinucleolin antibody, MS-3, revealed a major 110-kD band and faint lower molecular mass bands in the F3-bound material (Fig. 1 B, a). These bands were not present in eluates from the control peptide matrix. The faint bands are probably fragments of nucleolin, as they aligned with some of the several lower molecular mass bands detected by the antibody in a whole cell extract. These results show that the F3 peptide can specifically interact with nucleolin, suggesting nucleolin as a candidate receptor for F3.

We prepared antisera against nucleolin by immunizing rabbits with two different synthetic peptides from the human nucleolin sequence. Affinity-purified antibodies recognized a band that aligned with the 110-kD nucleolin band defined by the MS-3 antibody (Fig. 1 B, b). Like MS-3, the rabbit antibodies also detected smaller molecular mass bands that presumably represent nucleolin fragments. As expected, based on the fact that the immunizing peptides came from different regions of the nucleolin molecule, the sets of minor bands detected by each antibody did not overlap. Immunoblotting showed that the NCL3 antibody also recognizes a 110-kD protein in extracts of mouse cells. (Fig. 1 B, c).

Nucleolin is expressed at the cell surface

To serve as an F3 receptor, nucleolin would have to be present at the cell surface. Nucleolin is primarily known as a nuclear and cytoplasmic protein, but recent studies have shown that a cell surface form of nucleolin also exists (Said et al., 2002; Sinclair and O'Brien, 2002). To determine if F3-binding nucleolin in the MDA-MB-435 cells is ex-
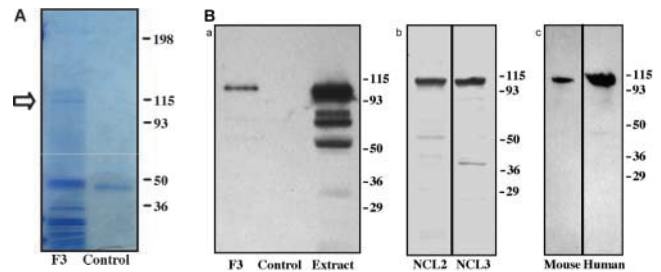

Figure 1. **Nucleolin binds to immobilized F3 peptide.** (A) SDS gel electrophoresis of Coomassie blue–stained proteins isolated from MDA-MB-435 cell extracts on F3 affinity matrix (F3) or control peptide matrix (Control). The arrow indicates a specific 110-kD band, which was identified as nucleolin by mass spectroscopy. (B) Immunoblotting of eluates from F3 and control affinity matrices with a monoclonal mouse antinucleolin antibody (a); immunoblotting of MDA-MB-435 cell extracts with polyclonal rabbit antinucleolin antibodies NCL2 and NCL3 (b); immunoblotting of extracts generated from the human cell line C8161 and the mouse cell line 4T1 with NCL3 (c). The F3 bound material (a, F3) contains full-length nucleolin and a faintly staining 75-kD band. In the original cell extract (a, Extract), the antibody recognizes full-length nucleolin at 110 kD, along with several faster migrating bands (presumably nucleolin fragments), including one at 75 kD. No antinucleolin reactive bands are detected in eluates from the control matrix (a, Control). Affinity-purified polyclonal antibodies NCL2 and NCL3 recognize a band that aligns with the 110-kD nucleolin band in human extracts, and NCL3 crossreacts with mouse nucleolin (c, Mouse). Both antibodies also detect smaller bands that presumably represent nucleolin fragments.

pressed at the cell surface, exponentially growing cells were biotinylated with a cell-impermeable biotin reagent, and cell extracts were subjected to affinity chromatography on immobilized F3. Two biotinylated bands at 110 and 75 kD specifically bound to F3 (Fig. 2 A, a). The surface biotinylated 75-kD band was stronger than the 110-kD band, whereas the opposite was true in the affinity chromatography, possibly because the cell surface expression or accessibility to biotinylation may be different for the two forms. Notably, the histones that bound to the F3 matrix from the cell extract did not become biotin-labeled in intact cells, but were the most prominent F3-binding bands from cell surface-biotinylated serum-starved cultures, which contain many dead cells (Fig. 2 A, b). No nucleolin band was detectable in the serum-starved cells, suggesting a lack of cell surface nucleolin expression.

Nucleolin was also detected at the cell surface of growing MDA-MB-435 in FACS® analysis using antibodies. Both the polyclonal NCL3 and monoclonal MS-3 antibodies bound to MDA-MB-435 cells, producing a distinct shift of the fluorescence peak relative to control IgG and nonsurface reactive antibody, respectively. That result was consistent in repeated experiments (Fig. 2 B, a and b). Gating for cells that were negative for propidium iodide uptake showed that the antinucleolin-positive cells were alive and their cell membranes were intact. We used the polyclonal antibody, which gave a stronger signal with the MDA-MB-435 cells than the monoclonal, to test cultured human umbilical vein endothelial cells (HUVECs) for cell surface nucleolin. A small shift in the fluorescence peak was observed (Fig. 2 B, c). These results show that nucleolin is expressed on the surface of the MDA-MB-435
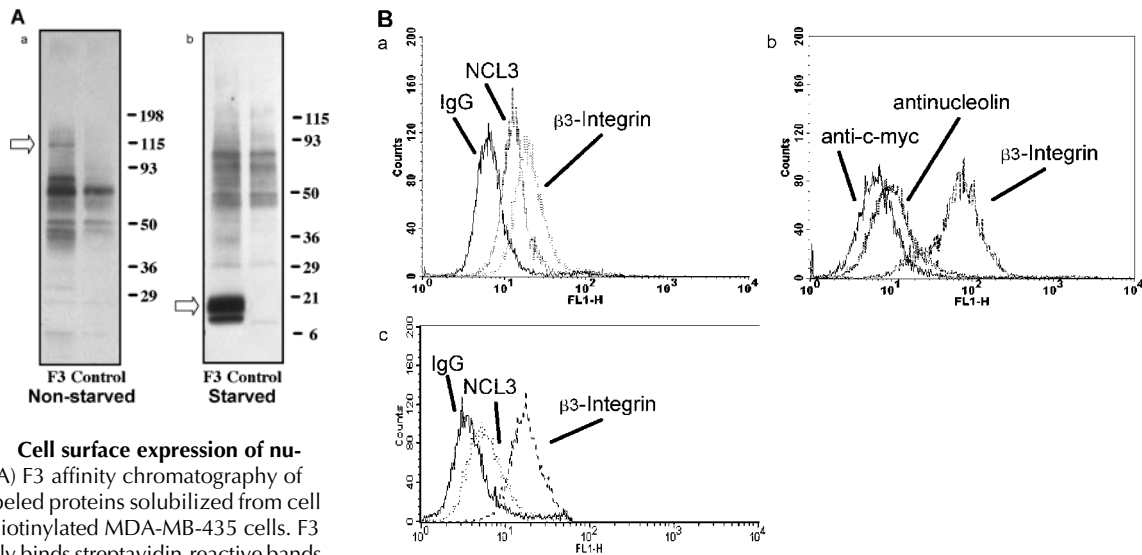
Figure 2. **Cell surface expression of nucleolin.** (A) F3 affinity chromatography of biotin-labeled proteins solubilized from cell surface–biotinylated MDA-MB-435 cells. F3 specifically binds streptavidin-reactive bands at 110 (arrow) and 75 kD in nonstarved cells (a). These bands are not detectable in serum-starved cells, but a set of low molecular mass bands is prominent (b, arrow). (B) FACS® analysis of antibody binding to MDA-MB-435 cells (a and b) and HUVECs (c). Propidium iodide–negative (living) cells were gated for the analysis. Polyclonal antinucleolin NCL3 (a and c) and monoclonal antinucleolin antibody MS-3 (b) cause a shift of the FACS® peak compared with controls (rabbit IgG and an isotype-matched monoclonal antibody with an unrelated specificity, respectively), indicating cell surface expression of nucleolin on the MDA-MB-435 and HUVECs. A positive control, an anti–β3 integrin, gives a strong shift, reflecting a high cell surface expression of this integrin subunit in both types of cells.

cells and that HUVECs may also express some cell surface nucleolin. Because the presence of many dead cells prevented a FACS® analysis on serum-starved MDA-MB-435 cells, we used immunostaining to study their subcellular nucleolin distribution. The NCL3 antibody stained the surface of MDA-MB-435 cells when the cells were actively growing, but there was no surface staining of these cells after they were rendered stationary by serum withdrawal (Fig. 3). Nuclear nucleolin was detected in permeabilized cells under both conditions. These results agree with the cell surface biotinylation data shown in Fig. 2 and suggest that cell surface expression of nucleolin is a characteristic of actively growing cells.

Antinucleolin antibodies inhibit internalization of F3 by cells

Nucleolin has been reported to shuttle between the cytoplasm and the nucleus (Shibata et al., 2002) and between the cell surface and the nucleus (Said et al., 2002). We used antibodies to study whether nucleolin is involved in the internalization and nuclear transport of F3. As shown previously (Porkka et al., 2002), fluorescein-labeled F3 was taken up by the MDA-MB-435 cells and localized in the cytoplasm and nucleus of 100% of the cells (Fig. 4, a–c). Coincubation of the cells with the NCL3 antibody inhibited the appearance of F3 in the cytoplasm and nucleus of the cells (Fig. 4, e–g). Instead, the antibody was internalized into the MDA-MB-435 cells and transported into the nucleus. NCL2, although it bound to the MDA-MB-435 cells, was not internalized and did not inhibit the cytoplasmic and nuclear localization of F3 (Fig. 4, i–k). Neither antibody affected the internalization of the cell-penetrating peptide from the Tat protein (Fig. 4, d, h, and l). These results indicate that F3 binds to cells and is internalized by them in a nucleolin-dependent manner that involves the NH2-terminal acidic domain of nucleolin.

Internalization of the F3 peptide into cultured cells is independent of heparan sulfates

Next, we determined whether glycosaminoglycans play a role in the internalization of F3 into cells. The F3 peptide is a highly basic peptide and, as such, has an affinity for negatively charged glycosaminoglycans. Previous studies have shown that binding to heparan sulfates can be sufficient for the internalization of a heparan sulfate–binding protein (Roghani and Mos-
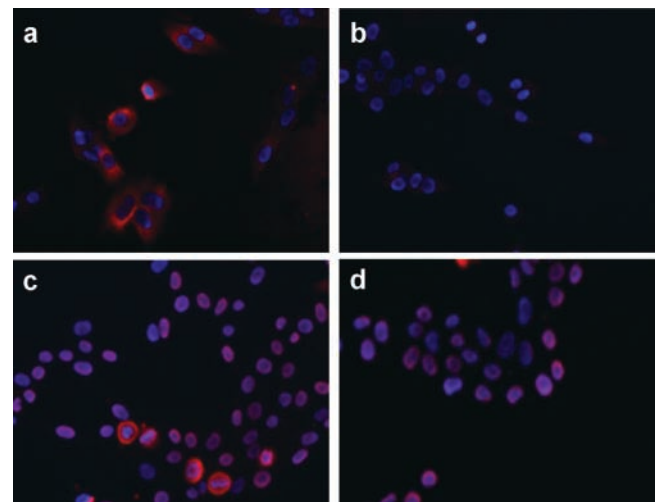
Figure 3. **Subcellular distribution of nucleolin in dividing and stationary cells.** MDA-MB-435 cells were cultured in standard culture media (a and c) or in media lacking serum (b and d). Nucleolin was detected using polyclonal NCL3 antibody in fixed cells without permeabilizing the cells (a and b) or after permeabilization with Triton X-100 (c and d). Nucleolin appears both on the surface and in the nuclei of the actively growing cells cultured in the standard media, but is exclusively nuclear in serum-starved cells.

Figure 4. **Antinucleolin antibodies inhibit F3 internalization by cells.** Exponentially growing MDA-MB-435 cells were incubated with 1 μM FITC-F3 or FITC-Tat peptide for 2 h at 37°C. FITC-F3 is internalized and transported into the nucleus (a, FITC-F3, green; b, red channel; c, merge). Coincubation with antinucleolin antibody NCL3 inhibits the cellular uptake and subsequent nuclear transport of the peptide (e, F3-FITC, green; f, NCL3, red; g, merge). NCL2 has no influence on uptake of F3 (i, F3-FITC, green; j, NCL2, red; k, merge). Internalization of FITC-Tat peptide (d) is not affected by NCL3 (h) or NCL2 (l). The antibodies were detected with Alexa-594 anti–rabbit IgG (red). Nuclei were stained with DAPI (blue). The images were obtained by confocal microscopy. Bars, 10 μm.
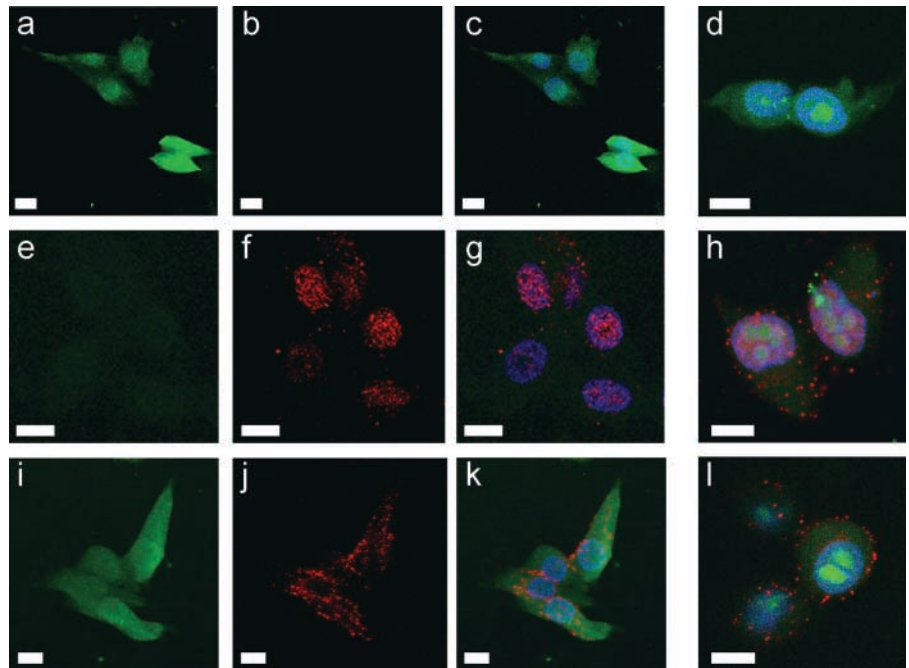
catelli, 1992). CHO cells that produce no glycosaminoglycans because of a mutated xylosyl transferase gene (pgsA-745 cells; Esko et al., 1985) internalized fluorescein-conjugated F3 and transported it into the nucleus as efficiently as the wild-type cells (Fig. 5). Neither cell type internalized a fluorescein-conjugated control peptide. Thus, glycosaminoglycans do not seem to be involved in the uptake of the F3 peptide into cells.
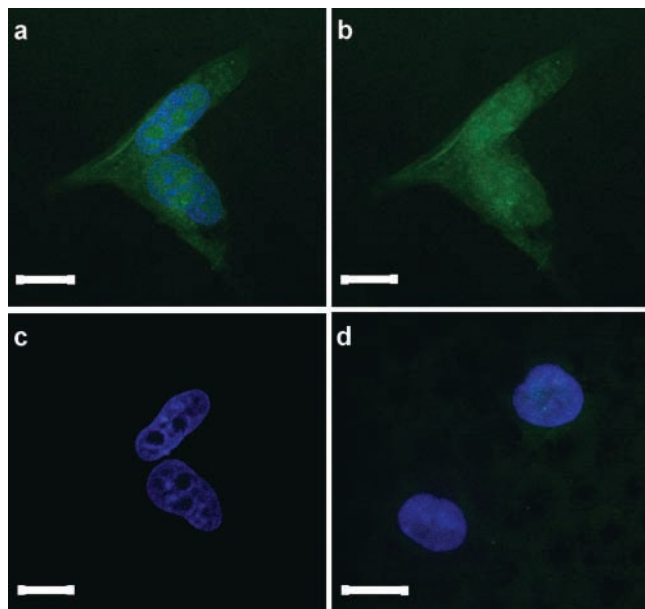
Figure 5. **Glycosaminoglycan-deficient cells bind and internalize F3.** FITC-F3 is internalized by the glycosaminoglycan-deficient pgsA-745 cells and transported into the nucleus. (a) pgsA-745 cells incubated with FITC-F3 and stained with DAPI to visualize the nuclei. (b and c) The same field as in panel a viewed separately for the F3 fluorescence (b) or the nuclear DAPI staining (c). (d) A FITC-labeled control peptide is not internalized by the pgsA-745 cells. The images were obtained by confocal microscopy. Bars, 10 μm.

Circulating antinucleolin antibodies selectively localize in angiogenic blood vessels

The F3 peptide, when expressed on the surface of phage, or labeled with fluorescein or quantum dots, selectively homes to tumor blood vessels and vessels in a matrigel angiogenesis
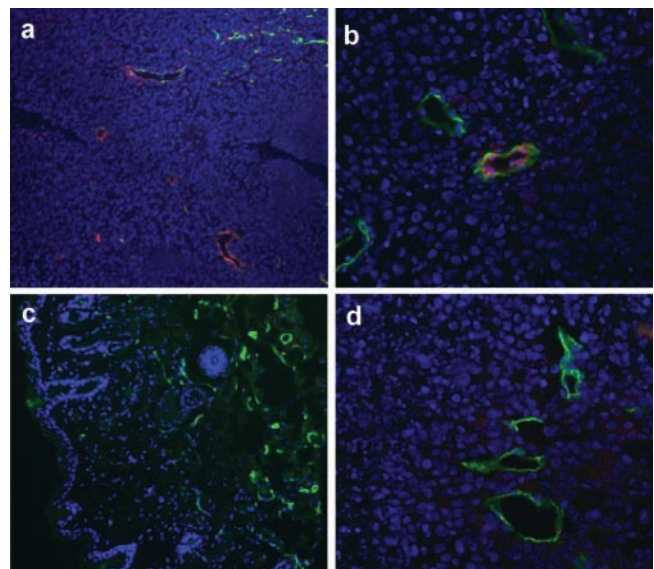
Figure 6. **Intravenously injected antinucleolin antibody accumulates in tumor blood vessels.** An affinity-purified rabbit antinucleolin antibody (NCL3) was injected into the tail vein of mice bearing MDA-MB-435 xenograft tumors. The tumor and various organs were removed 1 h after the injection, sectioned, and examined for the presence of rabbit IgG using Alexa-594 anti–rabbit IgG (red). Blood vessels were stained with anti-CD31 antibody (green), and nuclei were counterstained with DAPI (blue). The antinucleolin antibody has bound to tumor blood vessels (a and b), but is not seen in the skin (c). Rabbit IgG injected similarly as a control does not bind to tumor blood vessels (d).

Figure 7. **Cell surface nucleolin is expressed in angiogenic blood vessels.** Balb/c *nu/nu* mice were subcutaneously injected with matrigel supplemented with bFGF. 8 d later, an antinucleolin antibody (NCL3) or control 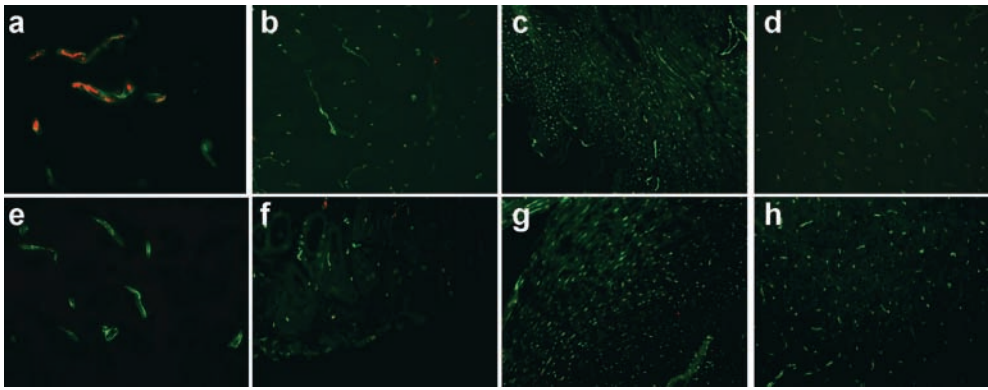IgG was injected into the tail vein of the mice. The matrigel plugs were removed 1 h after the injection, sectioned, and examined for the presence of rabbit IgG using Alexa-594 anti–rabbit IgG (red). Blood vessels were stained with anti-CD31 antibody (green), and nuclei were counterstained with DAPI (blue). The injected NCL3 colocalizes the blood vessel staining in the matrigel plugs (a), but no injected rabbit IgG is detected in the plugs (e). No specific NCL3 accumulation over the IgG control is seen in any of the tissues examined: b and f, skin; c and g, heart; or d and h, brain. b–d, NCL3; f–h, IgG.

model but not to normal blood vessels. (Akerman et al., 2002; Porkka et al., 2002; Joyce et al., 2003).

To determine whether antinucleolin would similarly accumulate in tumor vessels and/or tumor cells, we intravenously injected the NCL3 antibody into mice bearing MDA-MB-435 tumors. Tissues collected 60 min after injection showed selective accumulation of the antibody in tumor blood vessels (Fig. 6, a and b). No antibody was detected in association with the tumor cells. About 70% of the tumor vessels were positive for the antibody, whereas no positive vessels were seen in the blood vessels of the normal tissues tested (skin and lung; shown for skin subcutaneous tissue in Fig. 6, c). Purified rabbit IgG, injected as a control, was not detected in tumor blood vessels (Fig. 6, d).

Next, we examined the binding of antinucleolin antibodies to angiogenic blood vessels in a nonmalignant tissue (matrigel plugs impregnated with basic FGF as an angiogenesis inducer). Intravenously injected NCL3 antibodies selectively accumulated in 70% of the blood vessels of subcutaneously implanted matrigel plugs (Fig. 7, a). Control IgG was not detectable in the matrigel plug vessels (Fig. 7, e), and NCL3 was not detectable in the blood vessels of various control organs (Fig. 7, b–d). Thus, nucleolin appears to be selectively expressed on the cell surface of angiogenic blood vessels but not on blood vessels of other tissues in vivo.

Discussion

Here, we show that the tumor-homing F3 peptide, which binds to and is internalized by endothelial and tumor cells (Porkka et al., 2002), interacts with nucleolin. We also show that antinucleolin antibodies detect nucleolin at the surface of cultured tumor cells and endothelial cells of angiogenic vessels in vivo. These results support the previously proposed role for nucleolin as a shuttle molecule between the nucleus and the cell surface, and they define cell surface nucleolin as a novel vascular marker for angiogenic endothelium.

Several approaches were used to identify the binding molecule for the F3 peptide as nucleolin. First, nucleolin and histones were identified as the main cellular proteins that

specifically bound to immobilized F3 peptide. Cell surface labeling indicated that the bound nucleolin was derived from the surface of intact cells, whereas the histones were not labeled and, therefore, likely originated from dead cells. Second, inhibition of F3 uptake into cultured cells by an antinucleolin antibody that is internalized into the nucleus provides additional evidence for the specificity of the F3–nucleolin interaction and its occurrence in intact cells. Third, the specific binding of injected antinucleolin antibodies to tumor blood vessels extends the association of F3 binding and cell surface nucleolin expression to an in vivo animal model.

The nucleolin polypeptide consists of a negatively charged NH_2-terminal domain, an RNA-binding domain, and a COOH-terminal domain rich in RGG motifs. The main functions of nucleolin relate to rRNA maturation and ribosome assembly (Ginisty et al., 1999; Srivastava and Pollard, 1999). Although nucleolin was originally described as a nuclear and cytoplasmic protein, a number of studies show that it can also be expressed at the cell surface (Deng et al., 1996; Larrucea et al., 1998; Said et al., 2002; Sinclair and O'Brien, 2002). Recent results also ascribe additional functions to nucleolin as a shuttle protein between the cytoplasm and the nucleus (Borer et al., 1989; Yu et al., 1998), and between the cell surface and the nucleus (Schmidt-Zachmann and Nigg, 1993; Said et al., 2002; Shibata et al., 2002). The localization of nucleolin within the cell may be regulated by phosphorylation of its NH_2 terminus (Schwab and Dreyer, 1997). Our results provide additional evidence for the cell surface localization and shuttle function of nucleolin.

The expression of nucleolin at the cell surface seems to correlate with growth and metabolic activity of cells. Both the uptake of the F3 peptide and the staining of intact cells with antinucleolin antibodies were suppressed in serum-starved cells. This may be a proliferation-related effect. An association of cell surface nucleolin expression with cell proliferation in vitro has been described previously (Hovanessian et al., 2000). Other factors besides proliferation may contribute to the regulation of cell surface nucleolin expression. We found only modest levels of cell surface nucleolin on actively prolif-

erating endothelial cells in vitro, whereas antinucleolin binding to angiogenic endothelium was readily detectable in vivo. The differentiation state of the cells may be a factor contributing to nucleolin regulation, as cultured human leukemia-60 cells induced to differentiate into nonproliferating macrophages lose their ability to bind F3 (unpublished data). The restricted expression of cell surface nucleolin and the cell-type specificity of the expression may explain why some investigators have not been able to document the presence of nucleolin at the cell surface (Yu et al., 1998). A similar explanation may apply to the heterogeneity of the cell surface nucleolin expression in the vasculature of tumors and matrigel plugs; local variation in endothelial cell proliferation is likely to occur in angiogenic lesions in vivo.

F3-displaying phage selectively homes to tumor vasculature in vivo, and fluorescein-tagged F3 also binds to and is taken up by endothelial cells in tumor vasculature. However, the peptide also spreads to tumor cells, and it appears in a few individual nonvascular cells in the skin and the gut (Porkka et al., 2002). Intravenously injected antinucleolin antibody was only detected in angiogenic vessels of tumors as well as of matrigel plugs. The restricted distribution of the antibody resembles that of the phage, probably because the size of phage and antibody limit their access to tissues, whereas the relatively small molecular mass of the peptide conjugate (~5 kD) may permit wider distribution. Nonetheless, each of these reagents demonstrates the specificity of cell surface nucleolin for angiogenic vessels within the vasculature.

F3 is rich in basic amino acids and binds to cell surface heparan sulfate. However, our demonstration that CHO cells lacking heparan sulfate (and other glycosaminoglycans) internalize F3 excludes a direct role of heparan sulfate as the internalizing molecule. Indeed, binding and antibody inhibition studies show that F3 internalization is mediated by cell surface nucleolin. El-Sheikh et al. (2002) have described a peptide from the heparin-binding domain of vascular endothelial growth factor that selectively homes to tumor vasculature. The authors attributed the tumor homing to affinity of the peptide for heparan sulfate. It will be interesting to see whether this peptide might also bind to nucleolin.

The internalization of the F3 peptide and NCL3 antibody may reflect a physiological function of cell surface nucleolin. Midkine is a 13-kD cytokine that, like F3, contains a high proportion of basic amino acids (Said et al., 2002). It plays a role in neurite outgrowth and neuronal differentiation, and its mRNA is up-regulated in several human carcinomas (Tsutsui et al., 1993). The internalization of midkine by cells has been reported to be nucleolin dependent (Said et al., 2002), although lipoprotein receptor-related protein can also serve as the internalizing receptor for midkine (Shibata et al., 2002). The binding site for midkine in nucleolin has been localized to the RGG domain of nucleolin (Said et al., 2002), whereas our antibody inhibition results implicate the domain rich in acidic amino acids as the binding site for F3. Cell surface nucleolin may also be involved in the activities of basic FGF, which has been shown to bind to nucleolin in nuclear extracts (Bonnet et al., 1996). Thus, F3, midkine, and possibly basic FGF, might be internalized by a nucleolin-dependent mechanism, but distinct binding sites on nucleolin may exist to mediate the uptake.

A highly basic peptide derived from the HIV Tat protein also binds to cells and is internalized by them. The Tat peptide allows internalization of conjugated proteins and is commonly used as a cell-penetrating agent (Fawell et al., 1994; Langel, 2002). It is unlikely that the Tat peptide would use nucleolin for its internalization and nuclear transport. First, the internalization of Tat is independent of the cell type, even in vivo, whereas our results show that cell surface nucleolin is limited, it is expressed in angiogenic endothelium but not in the blood vessels in normal tissues. Second, treatment of cells with heparinase to remove heparan sulfates inhibits internalization of the Tat peptide (Suzuki et al., 2002), whereas we found that lack of heparan sulfates did not affect F3 uptake. Third, Tat internalization is independent of temperature and does not require energy, and several other cell-penetrating peptides are similar to Tat in this regard (Langel, 2002). In contrast, F3 uptake is blocked at $4°C$ (Porkka et al., 2002). Finally, our antibody inhibition data also suggest that Tat peptide internalization is independent of nucleolin because an antinucleolin antibody inhibited the uptake of F3 but not of the Tat peptide.

Our laboratory has recently described yet another type of a cell-penetrating peptide, LyP-1, which is also rich in basic amino acids (Laakkonen et al., 2002). This peptide specifically homes to the endothelium of tumor lymphatics and the tumor cells in certain, but not all, tumors. The internalization of this peptide is not affected by antinucleolin antibodies (unpublished data). Thus, several different internalization mechanisms for basic peptides appear to exist, both universal and cell-type specific.

Cell-penetrating peptides rich in basic amino acids are transported into the nucleus after internalization (Langel, 2002). This is also the case with F3 and LyP-1 (Laakkonen et al., 2002; Porkka et al., 2002). Nucleolin is thought to be responsible for the nuclear transport of midkine (Shibata et al., 2002), and the same may be the case with F3. It is also possible that the multiple basic amino acids in F3 form one or more independent nuclear localization signals.

The selective in vivo homing of the two nucleolin-binding reagents, the F3 peptide and the NCL3 antibody, to angiogenic blood vessels establishes cell surface nucleolin as a new angiogenesis marker. Tumor blood vessels undergo angiogenesis (Hanahan and Folkman, 1996) and have specific markers in common with other angiogenic vessels (Ruoslahti, 2002). Future studies will determine whether cell surface nucleolin might play a role in angiogenesis, possibly by binding and internalizing growth factors such as midkine and bFGF. The restricted expression of cell surface nucleolin in angiogenic vessels and in tumor cells in vivo, and its ability to internalize molecules bound to it, make nucleolin an attractive potential target for the development of agents for vascular therapy of tumors.

Materials and methods

Cells and antibodies

MDA-MB-435 cells were grown in RPMI 1640 medium with 10% FCS and 1% Glutamine Pen-Strep (Irvine Scientific). CHO-K1 and pgsA-745 cells were grown in αMEM Earle's salt with 10% FCS and 1% Glutamine Pen-Strep. The antibodies used were mouse monoclonal antinucleolin (IgG$_1$; MS-3; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal antibodies

raised against peptides synthesized according to the nucleolin sequence. NCL2 and NCL3 were raised against amino acids 43–51 and 221–232 of human nucleolin, respectively. The peptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits according to the manufacturer's instructions. Antibodies were affinity purified from the sera on the appropriate synthetic peptide immobilized on a SulfoLink Column (Pierce Chemical Co.; 2 mg of peptide covalently coupled/column). Bound antibodies were eluted with a glycine-hydrochloride buffer, pH 2.5, and neutralized with 1 M Tris-HCl, pH 8. Each antibody immunoblotted the same 110-kD nucleolin band in cell extracts as the monoclonal antinucleolin.

F3 affinity chromatography and mass spectroscopy

Affinity purification of nucleolin from MDA-MB-435 detergent extracts was performed as described previously (Christian et al., 2001b). In brief, 6×10^8 cells were pelleted and lysed in 60 ml of RIPA buffer (1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 1% protease inhibitor cocktail for mammalian cells; Sigma-Aldrich). The lysate was incubated with 20 μl F3 (AKVKDEPQRRSARL-SAKPAPPKPEPKPKKAPAKK) affinity matrix (2 mg of peptide covalently coupled to 1 ml of affigel 10). Control fractionation was performed on a 34–amino acid peptide that represents a scrambled version of F3. The matrix beads were washed three times with 0.025% Triton X-100, 50 mM Tris-HCl, pH 8.4, 150 mM NaCl, 1 mM CaCl$_2$, and 0.02% azide; washed twice with 25 mM Tris-HCl, pH 8.4, and 250 mM NaCl; and the bound proteins were eluted with 30 μl SDS gel sample buffer. The affinity-purified proteins were reduced with 50 mM DTT and separated on an 8–20% polyacrylamide gel and visualized by colloidal blue staining (Invitrogen). The molecular masses of the gel bands were determined by comparing to the standards in an Alphamanager instrument (Alpha Innotech Corp.). Bands that appeared in the F3 eluate, but not in the control, were cut out, digested with trypsin, and analyzed by mass spectroscopy using a matrix-assisted laser desorption ionization, time of flight instrument (model Voyager DE-PRO; Applied Biosystems) using an α-cyano-4-hydroxycinnamic acid/nitrocellulose matrix.

Immunoblot analysis

Cell extracts or affinity-purified samples were separated on an SDS-PAGE and transferred onto nitrocellulose membranes for 1 h at 100 V. The membranes were blocked overnight at 4°C with 5% milk powder in TBS-T (140 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.05% Tween) and incubated with mouse monoclonal or rabbit polyclonal antinucleolin antibody (10 μg/ml in TBS-T) for 1 h at RT. After extensive washing, the membranes were incubated with peroxidase-coupled rabbit anti–mouse or goat anti–rabbit antibody, and bound antibody was detected with ECL (Amersham Biosciences) and exposure to Biomax MR (Kodak).

Cell surface biotinylation

For cell surface expression analysis, MDA-MB-435 cells (5×10^6 cells) were washed three times with cold PBS on a cell culture plate and incubated with biotinylation buffer (20 mM HEPES, pH 7.45, 5 mM KCl, 130 mM NaCl, 0.8 mM MgCl$_2$, 1 mM CaCl$_2$, and 0.5 mg/ml EZ link Sulfo-NHS-Biotin; Pierce Chemical Co.) for 1 h at 4°C. After the removal of the reagent, the cells were washed three times with wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl$_2$, and 1 mM CaCl$_2$) and lysed in 1% Triton X-100 lysis buffer for 1 h. The lysates were centrifuged for 15 min at 15,000 g. F3 binding proteins were isolated by affinity chromatography as described above, separated on SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose membranes were incubated after blocking with ExtrAvidin–peroxidase conjugates diluted at 1:5,000 (Sigma-Aldrich) for 1 h at RT. Bands were detected after incubation with ECL reagent and exposure to Biomax MR.

FACS analysis of cell surface nucleolin

For FACS® analysis, MDA-MB-435 or HUVEC were detached with EDTA and 10^6 cells/sample were incubated with either polyclonal rabbit antinucleolin antibody NCL3 (10 μg/ml) or monoclonal antinucleolin antibody MS-3 (Santa Cruz Biotechnology, Inc.; 10 μg/ml) for 45 min on ice. Cells were washed with ice-cold PBS and incubated with Alexa-488 (Molecular Probes) secondary antibody (1:50 in PBS). As a negative control, the cells were incubated with 10 μg/ml of rabbit IgG or with 10 μg/ml monoclonal anti–c-myc antibody (Santa Cruz Biotechnology, Inc.), followed by the secondary antibody. As a positive control, the cells were incubated with 10 μg/ml of mouse monoclonal anti–β$_3$-integrin (CBL 479; Cymbus Biotechnology Ltd.). The antibody-treated cells were washed and resuspended in 50 μl PBS containing 2 μg/ml propidium iodide to distinguish between live and dead cells, and 10,000 cells per sample were analyzed using a FACSCalibur flow cytometer.

Detection of peptides and antibodies in cells and tissues

For internalization experiments, cells were incubated with 1 μM of fluorescein-conjugated peptide for 2 h at 37°C. The cells were washed with PBS, fixed with 4% PFA in PBS, and analyzed by confocal microscopy. The cell-penetrating basic peptide from the human immunodeficiency virus Tat protein (GRKKRRQRRR; Fawell et al., 1994) was used as a positive control in the internalization experiments. To detect nucleolin, cells were fixed with 4% PFA in PBS and stained with 10 μg/ml antinucleolin antibodies either directly or after permeabilization with Triton X-100. Bound antibodies were detected with Alexa-594–labeled anti–rabbit antibody (Molecular Probes) and visualized by fluorescence microscopy.

In vivo distribution of circulation-accessible cell surface nucleolin was examined in mice bearing xenograft tumors or basement membrane (matrigel) plugs. Xenograft tumors were generated by subcutaneously injecting exponentially growing MDA-MB-435 human breast cancer cells (10^6 cells in 200 μl of culture media) into the mammary fat pad area of 2-mo-old Balb/c nu/nu mice (Animal Technologies). The animals were used for experiments 8 wk after injection. Nontumor angiogenesis was studied in matrigel plugs (Fulgham et al., 1999; Ngo et al., 2000). 2-mo-old Balb/c nu/nu mice were subcutaneously injected with 100 μl of Matrigel (Becton Dickinson) at two or three locations in the abdominal area. Each 100-μl plug contained 100 ng of recombinant human bFGF as an angiogenesis stimulant (R&D Systems). The animals were used for antibody injection experiments 8 d after the implantation.

In vivo distribution of antibodies was studied by intravenously injecting mice with 200 μg of polyclonal rabbit antinucleolin antibody or rabbit IgG. 1 h after the injection, the mice were anesthetized, perfused through the heart with 10 ml PBS, and killed by infusing 10 ml of 4% PFA in PBS. Tumors or matrigel plugs, along with various control tissues were removed, fixed in 4% PFA, and frozen in OCT embedding medium (Tissue-Tek). All procedures were performed under anesthesia induced by intraperitoneal injection of 2,2,2-tribromoethanol (Avertin) at a dosage of 0.4–0.75 mg/gram of body weight (500–700 μl/mouse). All animal experiments were approved by the Animal Review Committee of the Burnham Institute.

For histological analyses, 5-μm sections were cut. The injected rabbit antinucleolin antibody and anti-CD31 antibody (10 μg/ml; BD Biosciences) applied on the tissue sections were detected with Alexa-594– and Alexa-488–conjugated secondary antibodies, respectively. The sections were examined under an inverted fluorescent microscope (Nikon) or a confocal microscope (Bio-Rad Laboratories). Nuclei were counterstained using DAPI (Vector Laboratories).

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