Nucleosomes Bind Fibroblast Growth Factor-2 for Increased Angiogenesis In vitro and In vivo

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Abstract

Solid tumors often display sites of necrosis near regions of angiogenesis in vivo. As tumor cell necrosis would result in the release of nucleosomes into the extracellular environment, we explored the potential role of nucleosomes in the promotion of angiogenesis. Data indicate that nucleosomes acted similar to heparin and bound to several heparin-binding, proangiogenic factors [i.e., fibroblast growth factor (FGF)-1, FGF-2, vascular endothelial growth factor, and transforming growth factor-β1]. Nucleosomes modestly enhanced FGF-2 growth of human umbilical vein endothelial cells when grown in restricted media as well as increased human umbilical vein endothelial cell migration and primitive blood vessel tube formation in vitro. On s.c. injection in mice, nucleosomes aided FGF-2 in promoting angiogenesis. These results suggest that nucleosomes released from dying tumor cells aid in the formation of blood vessels and may provide a novel means by which tumor cells increase angiogenesis.
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Introduction

Angiogenesis, or the formation of new blood vessels, is an essential component for solid tumor cell survival and tumor metastasis (1). Newly formed blood vessels provide both an increased availability of oxygen and nutrients to the burgeoning tumor mass and an exit route for tumor cells to enter the circulation (2, 3). However, despite extensive angiogenesis, tumor vascularization is often poorly organized and marginally functional due to structural abnormalities of the tumor mass (4). Consequently, marked variations in regional or temporal oxygen and waste exchange exist, which lead to the induction of hypoxia or increased toxic metabolites and the development of apoptosis and necrosis (5). Necrosis, a process that represents the final stage of cell death, results in the release of nucleosomes into the extracellular space as evidenced by increased amounts of nucleosomes in the blood of cancer patients (6-8). Nucleosomes constitute the primary subunits of eukaryotic chromatin and are composed of a negatively charged double-stranded DNA wrapped around an octameric motif formed from positively and negatively charged histones (9).

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Interestingly, on examination of melanoma, glioblastoma, and pancreatic tumors, one often observes sites of tumor necrosis juxtaposed to areas exhibiting elevated angiogenic growth factor expression and neoangiogenesis (10, 11). The two more common angiogenic factors used by various cancer cells for growth and metastatic progression are basic fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF; ref. 12). FGF-2 and several isoforms of VEGF are members of a larger family of proteins that bind serum heparin and cell surface heparan sulfate proteoglycans (13). The ability of FGF-2 and these VEGFs to bind to heparan moieties was found essential for their maximal binding to their receptor and results in increased epithelial cell proliferation, migration, and blood vessel formation (14-16).

As sites of tumor necrosis and angiogenesis appear physically linked and nucleosomes may share the charge characteristics of heparin or heparan sulfate, we explored whether nucleosomes could mimic heparin and serve as a unique vehicle to which heparin-binding angiogenic factors bind and enhance angiogenic signaling in vitro in endothelial cells and increase angiogenesis in vivo.

Results

Nucleosomes Bind to Heparin-Binding Angiogenic Factors

FGF-2, along with several other angiogenesis-related growth factors and cytokines, interacts with negatively charged heparin through a common set of basic amino acid clusters (17, 18). As nucleosomes contain clusters of positively and negatively charged moieties and thus may mimic heparin, we examined whether nucleosomes could bind to FGF-2. As shown in Fig. 1A, nucleosomes strongly bound to FGF-2-coated ELISA plate wells (2-fold; Fig. 1A, *FGF*) but did not bind to wells coated with BSA (Fig. 1A, *BSA*), indicating that nucleosomes recognized FGF-2. To determine whether nucleosomes recognized FGF-2 in a heparin-like manner, we challenged potential nucleosome-FGF complexes with soluble heparin. Results showed that soluble heparin blocked nucleosomes from binding to FGF-2 by 94% (Fig. 1A, *FGF + Hep*). We also noted that nucleosomes did not bind heparin-coupled BSA-coated wells (Fig. 1A, *Hep-BSA*), which would suggest that the ability of heparin to block nucleosome binding to FGF-2 was likely due to heparin interaction with FGF-2 and not with nucleosomes.

Examination of angiogenesis-related heparin-binding growth factors [FGF-1, FGF-2, VEGF, and transforming growth factor (TGF-β1)] or two non-heparin-binding growth factors (i.e., insulin growth factor-I and epidermal growth factor) for their ability to bind to nucleosomes revealed that nucleosomes

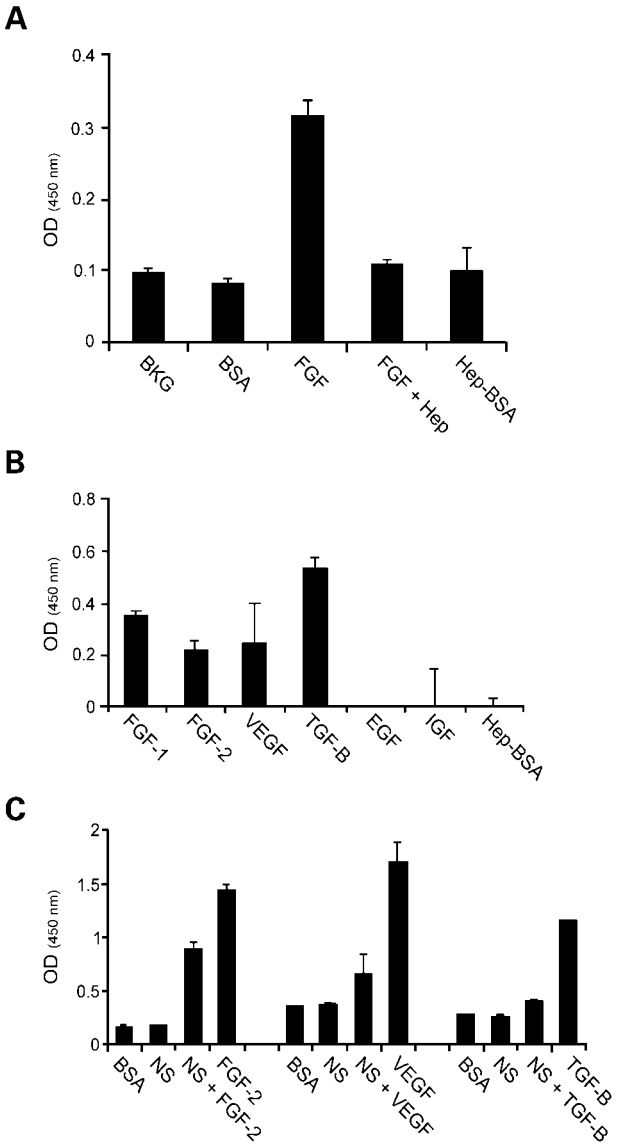

FIGURE 1. Nucleosomes bind heparin-binding angiogenic molecules. **A.** Triplicate wells coated with BSA, FGF-2, or heparin-coupled BSA (*Hep-BSA*) were incubated with nucleosomes and washed as outlined in Materials and Methods, and bound nucleosomes were detected using MAB3037. For heparin competition experiments (*FGF + Hep*), heparin sulfate was added to FGF-2–coated wells 1 hour prior to and during incubation of wells with nucleosomes. **B.** Triplicate wells coated with FGF-1, FGF-2, VEGF, TGF-β1, epidermal growth factor, insulin growth factor, or Hep-BSA and incubated with nucleosomes were washed, and bound nucleosomes detected with MAB3037. **C.** Triplicate wells coated with nucleosomes (*NS*) were incubated with rFGF-2, $rVEGF_{165}$, or rTGF-β1 followed by incubation with anti-FGF-2, anti-VEGF, or anti-TGF-β1 monoclonal antibody. Bound antibodies were detected with biotin-labeled goat anti-mouse and horseradish peroxidase–coupled streptavidin. Results in **A** include the background reactivity for wells coated with BSA (*BKG*). Results in **B** were plotted following the subtraction of BSA background activity. For **C**, BSA or purified growth factor–coated wells served as antibody background and positive well controls, respectively. *Columns,* mean absorbance; *bars,* SD.

bound preferentially 2- to 5-fold to the heparin-binding proteins (FGF-1, FGF-2, VEGF, and TGF-β1) but not to the non-heparin-binding proteins (epidermal growth factor, insulin growth factor-I, or heparin-coupled BSA; Fig. 1B).

To further verify that nucleosomes bound to these heparin-binding growth factors, we performed a converse set of experiments wherein nucleosomes were initially coated onto ELISA plate wells followed by a challenge with FGF-2, VEGF, or TGF-β1. Additionally, as we would use these nucleosome preparations in various in vitro and in vivo biological experiments, we also wished to exclude the possibility that the nucleosome stocks contain these heparin-binding growth factors. The results shown in Fig. 2C indicated that the purified nucleosome stocks did not elicit any significant reactivity to antibodies specific for FGF-2, VEGF, or TGF-β1 (Fig. 1C, 1.7%, 7%, and 0% increased absorbance, respectively, for *NS* vs. *BSA*) and were therefore unlikely to contain these three growth factors. Further support for the notion that the nucleosome preparations were free of FGF-2, VEGF, or TGF-β1 comes from the reported findings that Jurkat cells do not express detectable FGF-2 or VEGF proteins and express only low levels of TGF-β1 mRNA transcript (19, 20).

On the other hand, our experiments indicated that when nucleosomes were challenged with FGF-2, VEGF, or TGF-β1, we observed a 5-, 2-, and 0.5-fold increase, respectively, in FGF-2, VEGF, and TGF-β1 antibody reactivities (Fig. 1C, *NS* vs. *NS* + growth factor), indicating that these three angiogenic factors recognized plate-bound nucleosomes. While the

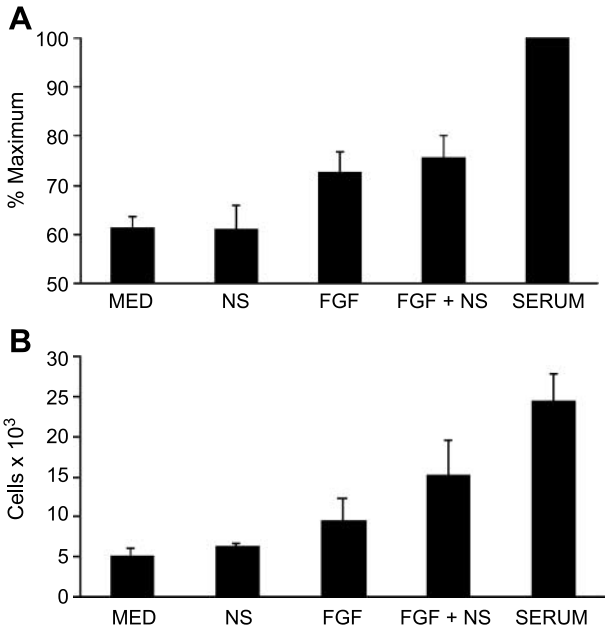

FIGURE 2. Growth of HUVEC following nucleosome treatment. **A.** Triplicate cultures of HUVEC seeded at 1×10^3 cells in 96-well plates were cultured in serum- or supplement-free medium (*MED*) containing nucleosomes (*NS*), FGF-2 (*FGF*), or complete medium (*SERUM*). After 36 hours of culture, cells were pulsed for 4 to 12 hours with 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt for spectroscopic measurement of 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt catabolism. **B.** HUVECs were seeded at 5×10^3 in 24-well culture plates in serum- or supplement-free medium containing nucleosomes, FGF-2, or complete medium. After 72 hours, cells were removed from the culture plate with trypsin and counted. *Columns,* percentage maximum growth (**A**) or total cell number per well (**B**) for eight and three independent experiments, respectively; *bars,* SE. Serum-containing cultures were given an arbitrary percentage value of 100.

increased nucleosome binding activity exhibited for each of the individual growth factors in Fig. 2C differed from that seen when individual growth factors were bound to ELISA plate wells and challenged with nucleosomes (Fig. 1B) and could be attributed to antibody obstruction following growth factor-nucleosome interaction, these results indicated that nucleosomes possess the ability to bind to FGF-1, FGF-2, VEGF, and TGF-β1 and thus suggest that nucleosomes may serve, like heparin, to maximize growth factor-receptor interaction (13). Another possibility is that nucleosomes may act as a stabilizing agent or carrier for the deposition of these growth factors in neighboring tumor tissue (16).

Nucleosomes Promote FGF-2 Cell Growth

As a result of tumor cell hypoxia, starvation, or stress, angiogenic factors such as FGF-2 can be expressed in tumor tissues. The expression of FGF-2 in turn promotes the growth and differentiation of tumor endothelial cells (12). Testing of endothelial cells under growth-restricted conditions revealed no significant increase in cell growth or cell number when culture medium was supplemented with nucleosomes (Fig. 2A and B, *MED* vs. *NS*); however, when given growth-limiting amounts of FGF-2 and an optimized amount of nucleosomes, human umbilical vein endothelial cells (HUVEC) increased their cell metabolism to 75% of maximum growth as compared with cells grown only in restricted medium (61% of maximum growth; Fig. 2A, *MED* vs. *FGF + NS*). Cell number increased 3-fold compared with cell growth in restricted media (Fig. 2B, *MED* vs. *FGF + NS*). Depending on the cell type and experimental conditions, prior studies indicate that heparin increases FGF-2 cell proliferation by 2- to 10-fold (14, 21-23). Our results indicate that nucleosomes alone did not promote HUVEC growth and had a modest growth and proliferative effect when given to HUVEC with FGF-2 (Fig. 2A and B, *NS* and *FGF* vs. *FGF + NS*).

Nucleosomes Stimulate Migration and Invasion of HUVEC

An important initial step in angiogenesis is the migration and invasion of endothelial cells to sites of needed vessel formation (24). Migrating endothelial cells must break and traverse their basement membrane through the activation of proteolytic enzymes. Boyden chambers have been employed previously with success to screen potential angiogenic factors by measuring their effect on HUVEC migration and invasion (25, 26). We measured the effects of nucleosomes on epithelial cell migration using Boyden chambers and observed that nucleosomes increased HUVEC migration to the lower chamber by 56% as compared with medium (Fig. 3, *MED* 108 ± 4 cells vs. *NS* 168 ± 14 cells; $P = 0.06$). When nucleosomes were tested in conjunction with FGF-2, migration of HUVEC into the bottom chamber increased by 87% (Fig. 3, *MED* 108 ± 4 cells vs. *FGF + NS* 202 ± 12 cells; $P = 0.008$). The increased levels of HUVEC migration seen for nucleosomes or nucleosomes plus FGF-2 are comparable with that seen when medium was supplemented with the singlequot cocktail of angiogenic factors (70% increase for ANGIO+, *MED* 108 ± 4 cells vs. ANGIO+ 185 ± 5 cells). Under these

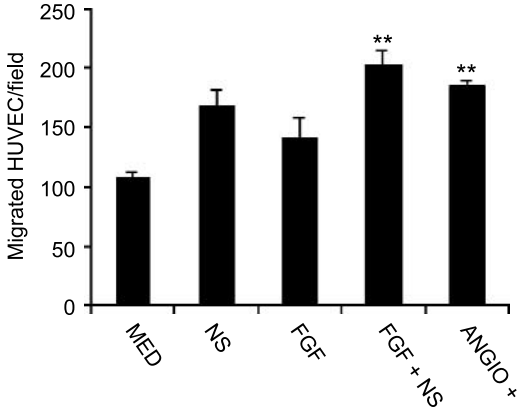

FIGURE 3. Nucleosomes stimulate HUVEC migration. Nucleosomes (*NS*) or FGF-2 (*FGF*) in supplement-free EGM-2 medium or complete EGM-2 medium (*ANGIO+*) was added to the lower Boyden chamber. HUVECs were added to the upper chamber and cultured for 4 to 5 hours. The underside of the upper chamber filter was photographed in situ for three separate fields and adherent cells were counted. *Columns,* mean cell values for three independent experiments; *bars,* SE. **, $P < 0.01$ for MED versus test set obtained by the two-tailed, paired Student's *t* test.

experimental conditions, the increased percentage or the actual number of HUVEC that migrated to the lower chamber when treated with nucleosomes or FGF plus nucleosomes are comparable with studies by Malinda et al. (27, 28) who studied induction of endothelial cell migration and angiogenesis by thymosin α_1 and β_4.

Nucleosomes Promote Vascular Remodeling of HUVEC

HUVEC are known to form primitive blood vessels or tube structures in vitro following exposure to angiogenic factors such as FGF-2 (29). Inducers of early-stage angiogenesis promote endothelial cells to undergo vascular tube formation in vitro when grown in a three-dimensional lattice consisting of Matrigel. The ability of purified nucleosomes to induce HUVEC tube formation in vitro was assessed as described by Malinda et al. (27). Representative microscopic fields were photographed and the percentage of tube formation or angiogenesis following various nucleosome treatments was determined. A representative field and a summary graph from three independent experiments are portrayed in Fig. 4A and B, respectively. HUVEC grown in Matrigel without supplements showed 47 ± 13% tube formation as compared with Matrigel supplemented with a cocktail of angiogenic factors (Fig. 4B, *MG* vs. *ANGIO+*). In contrast, HUVEC grown in Matrigel supplemented with nucleosomes or FGF-2 showed tube formation angiogenesis of 107 ± 5% and 100 ± 9% tube formation, respectively, compared with ANGIO+ medium (Fig. 4B, *NS* and *FGF* vs. *ANGIO+*). This increase in HUVEC tube formation was enhanced if Matrigel was supplemented with both FGF-2 and nucleosomes. Together, these two elements showed an enhancement of 120 ± 7% in tube formation as compared with ANGIO+ medium (Fig. 4B, *FGF + NS*). Taken together, our results support the idea that nucleosomes constitute an angiogenic-promoting factor, which may act either alone or in conjunction with other angiogenic factor such as FGF-2 to enhance normal epithelial cell formation of vascular structures in vitro.

Nucleosomes Promote Vascularization In vivo

To assess the ability of nucleosomes to promote angiogenesis in vivo, we used the Matrigel plug assay (30). Ten days postinjection, Matrigel plugs were excised, photographed, and assayed for hemoglobin content. Figure 5A shows a representative set of Matrigel plugs for each treatment (arbitrarily designated 1 to 3) taken from a treatment set of six to eight mice, illustrating the range of induced blood vessel formation in vivo. The level of blood vessel formation within the plugs can be judged by the degree of red color in the Matrigel plug as well as detection of concentrated areas of RBC and vascular structures in the Matrigel plug. We observed that Matrigel or Matrigel supplemented with heparin contained little or no red color, indicating a lack of angiogenesis. Matrigel containing nucleosomes or FGF-2 displayed sites of angiogenesis as indicated by the presence of blood vessel structures. The amount of angiogenesis in Matrigel containing both FGF-2 and heparin or Matrigel containing both FGF-2 and nucleosomes increased significantly as noted by an increase in the number of red Matrigel plugs and the higher number of vascular structures (Fig. 5A).

Further microscopic examination of the Matrigel plugs showed that plugs supplemented with nucleosomes and FGF drew cells from the surrounding tissue into the Matrigel to form new vasculature networks. These new vessels were abundantly filled with intact RBC, indicating that nucleosomes and FGF-2 formed functional vasculature with blood circulation inside the Matrigel plug (Fig. 5B).

To quantify the amount of angiogenic activity associated with the various treatments, hemoglobin content, a surrogate marker for angiogenesis, was assessed in our Matrigel plugs (31, 32). As shown in Fig. 5C, individually, heparin, nucleosome, or FGF-2 treatment did not significantly increase the amount of hemoglobin content as compared with Matrigel; however, combining

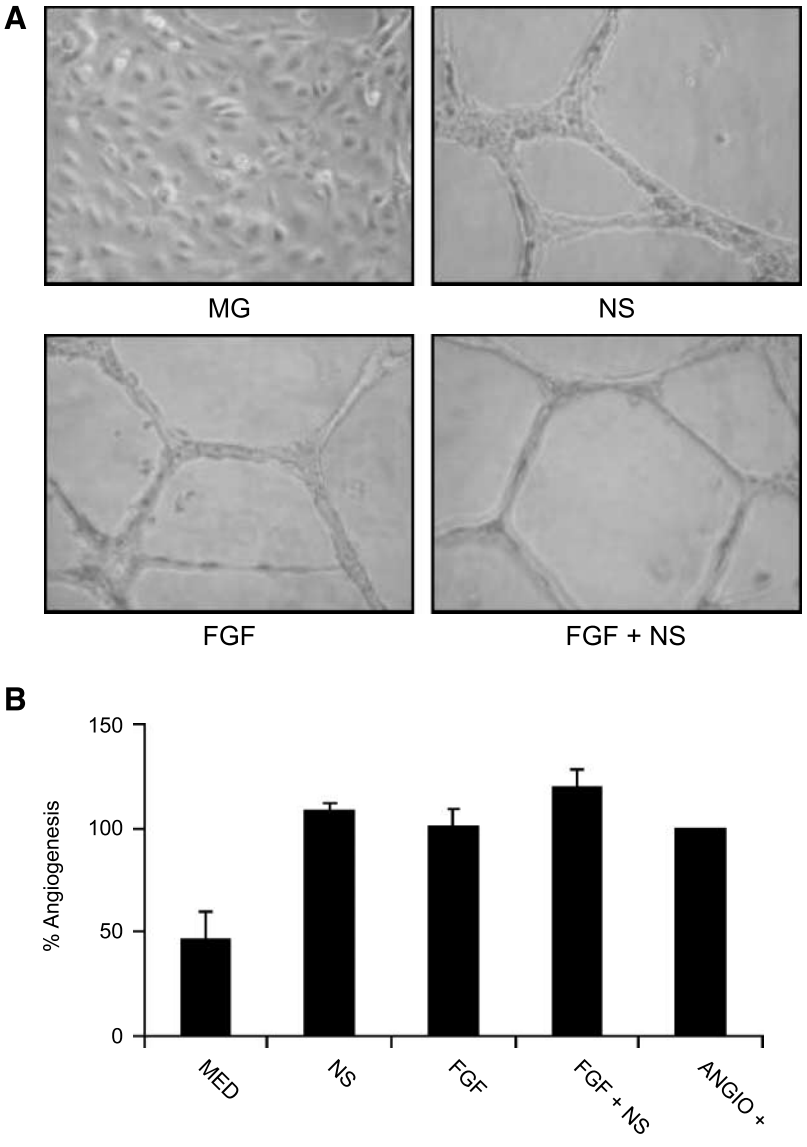

FIGURE 4. Nucleosomes stimulate HUVEC tube formation in vitro. **A.** Representative photomicrographs of HUVEC seeded on Matrigel (*MG*) in the presence of nucleosomes (*NS*), FGF-2 (*FGF*), or complete EGM-2 media (*ANGIO+*). **B.** Photomicrographs of three separate cell fields per treatment for three independent experiments were taken after 18 hours of cell culture. *Columns,* percentage angiogenesis; *bars,* SE. ANGIO+ cultures were given arbitrary percentage values of 100.

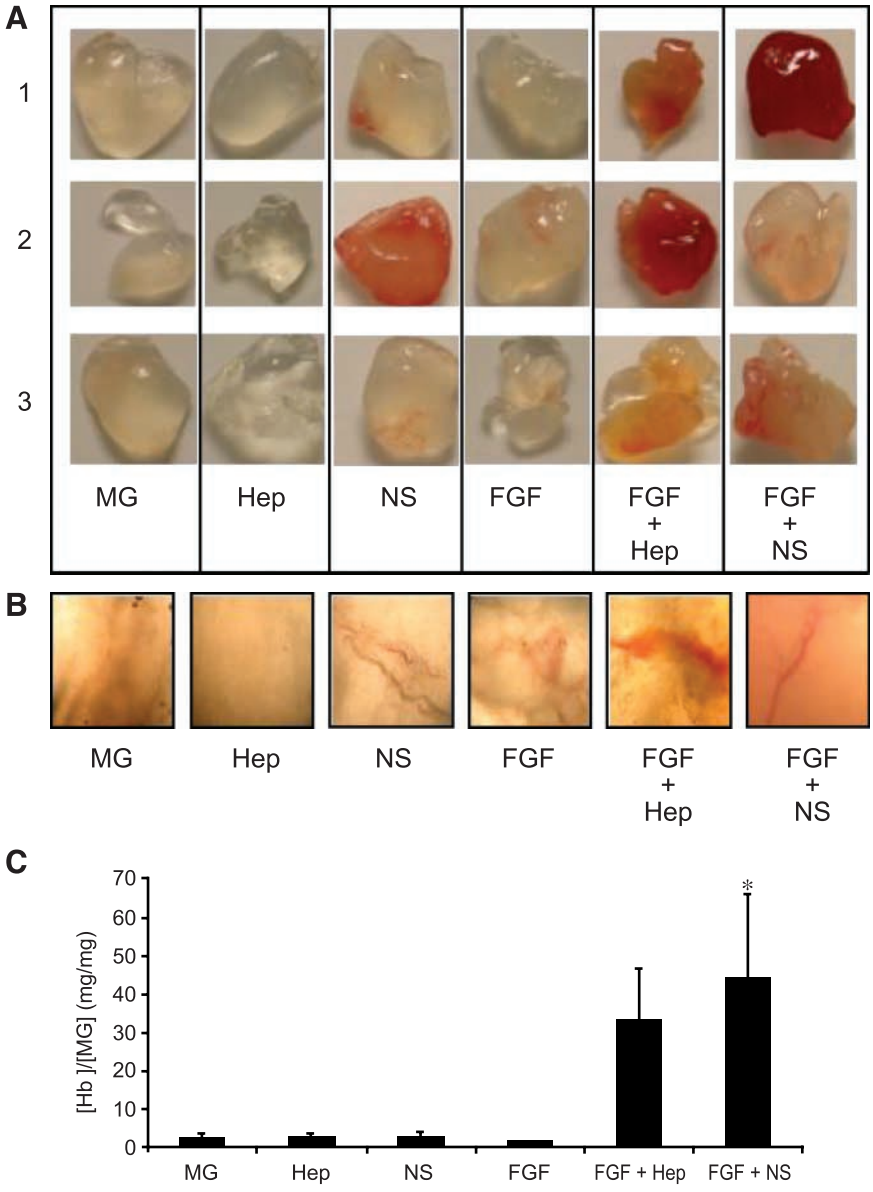

FIGURE 5. Nucleosomes promote angiogenesis in vivo. Eight-week-old mice were s.c. injected with Matrigel (*MG*) into the ventral midline (two paired *MG* plugs per mouse per eight mice) containing heparin (*Hep*), FGF-2 (*FGF*), or nucleosomes (*NS*). **A.** Representative photographs of *MG* plugs excised 10 days postinjection from three mice (arbitrarily designated *1* to *3*). **B.** Photomicrographs of *MG* plugs revealing vascularization following *Hep*, *NS*, or *FGF-2* treatment. **C.** Hemoglobin content of *MG* plugs obtained for the *MG*-injected animals. *Columns,* mean value for two independent experiments and a total of 8 plugs per treatment; *bars,* SE. *, $P < 0.05$ for *MG* versus test set obtained by the two-tailed, paired Student's *t* test.

both FGF-2 and nucleosomes resulted in a 37-fold increase in Matrigel plug hemoglobin content versus Matrigel alone ($P = 0.02$). These results coupled with our in vitro findings strongly support the notion that nucleosomes worked in conjunction with FGF-2 to markedly promote angiogenesis in vivo.

Discussion

Angiogenesis has been studied for many years in the context of wound healing and various diseases such as diabetes, arteriosclerosis, ischemic heart disease, and cancer (33). Traditional angiogenic factors are composed of various cytokines and growth factors (34). Our results provide evidence to support the notion that nucleosomes represent a new angiogenic component. Nucleosomes appeared to mimic heparin and bind several angiogenic factors; like heparin, nucleosomes were shown to augment FGF-2 angiogenic cell signaling in vitro and in vivo (Figs. 1-5). Furthermore, we observed that addition of only nucleosomes to HUVEC cultures increased both in vitro cell migration and tube formation (Figs. 4 and 5, *NS*). Although our nucleosome preparations revealed no significant protein bands other than histones or the presence of FGF-2, VEGF, or TGF-β1 when tested by SDS-PAGE or ELISA (Figs. 1C and 6B), and we cannot rule out the possibility that the nucleosome preparation contained alternate angiogenic factors, we have recently observed that nucleosomes stimulated the expression of the angiogenic growth factors interleukin (IL)-6 and IL-8 in HUVEC (35). Other laboratories have also noted an increase in IL-6 expression in peripheral blood mononuclear cells following nucleosome exposure (36). Given that IL-6 and IL-8 are crucial for endothelial cell survival and promote angiogenesis (37-39), our current observations noting the induction of HUVEC migration and tube formation following only nucleosome treatment may be due to the induction of IL-6 and IL-8.

FGF-2 and other prominent angiogenic factors belong to a family of molecules that interact with heparin and heparan sulfate to form dimers and higher-order oligomers (14). For FGF-2, coupling to heparin results in a 2- to 10-fold amplification of receptor signaling as compared with monomeric FGF-2 (21-23). Heparin binding to FGF-2 prolongs FGF-2 biological half-life by shielding it from hostile environmental conditions or proteases (21). Prior studies have shown that the polyionic compounds dextran sulfate and protamine, at concentrations equimolar or 10-fold in excess to FGF-2, respectively, acted like heparin and bound FGF-2 (40, 41). In doing so, both augmented the growth of fibroblasts or endothelial cells by FGF-2. Because nucleosomes exhibit charged surface moieties similar to these molecules or to heparin and thus might mimic heparin or heparan sulfate proteoglycans, we examined whether FGF-2 and other heparin-binding proteins bind to nucleosomes and whether nucleosomes augmented HUVEC response to FGF-2. Our ELISA results indicated that nucleosomes bound heparin-binding proteins FGF-1, FGF-2, VEGF, and TGF-β1 but not non-heparin-binding proteins insulin growth factor-I or epidermal growth factor (Fig. 2B). Further, heparin successfully competed with nucleosomes for FGF-2 binding sites (Fig. 2A), suggesting that the nucleosome recognition site on FGF-2 may be at or near the heparin-binding site of FGF-2 (17). Examination of HUVEC in vitro growth response to nucleosomes and FGF revealed that nucleosomes increased the ability of FGF-2 to increase HUVEC proliferation (Fig. 3). The molar concentration of nucleosomes used to support HUVEC growth was in agreement with the concentration of heparin previously reported to augment FGF-2 biological activity (240 pmol/L for nucleosomes vs. 740 pmol/L for heparin, assuming an average molecular mass for heparin of 13,500 daltons; ref. 42).

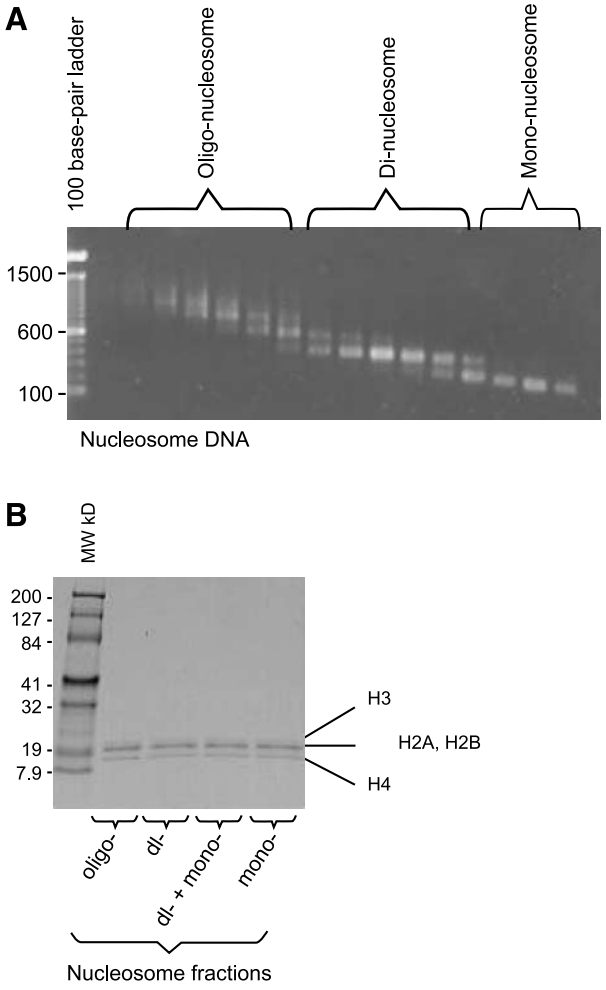

FIGURE 6. Characterization of purified nucleosomes. (**A**) Agarose and (**B**) SDS-PAGE gel of nucleosome purification. **A.** The characteristic 120-bp DNA fragments typical of purified monomeric nucleosomes (120 bp), dimeric nucleosomes (240 bp), and oligomeric nucleosomes (<480 bp). Two micrograms of nucleosomes per lane from pooled nucleosome fractions were resolved in a 10% to 20% SDS-PAGE gradient gel and stained with Bio-Rad Blue stain. **B.** The characteristic histone components of nucleosomes (*H2A*, *H2B*, *H3*, and *H4*) devoid of contaminating cellular protein.

Tumors display areas of significant apoptosis and necrosis that is often found intimately coupled to sites expressing angiogenic growth factors and active angiogenesis (5, 10, 11). In this microenvironment, nucleosomes from the dying cells might promote angiogenesis by binding to angiogenic molecules and augmenting angiogenic factor cell signaling. Unlike other members of the FGF family, FGF-1 and FGF-2 do not have classic signal sequences (43). In quiescent cells, FGF-2 immunoreactivity is typically seen in the nucleus, but, on cell activation or stress, FGF-2 is newly detected on the cell surface or in the surrounding medium. The method by which FGF-2 is transported to the exterior of the cell is still under investigation; however, FGF-2 is released following cell death or after non-lethal membrane disruption (44, 45). On the basis of our findings, we speculate that nucleosomes released from dying cells or present in necrotic tumor tissue would recognize FGF-2 either inside the cell during late stage cell death or when present in the surrounding tumor stroma (5-7). By binding to FGF, nucleosomes might then serve to prolong FGF-2 biological half-life or aid in its transport to adjoining stromal epithelium (21).

In summary, our results suggest that nucleosomes have the potential to promote the growth and expansion of cancer tissue through the recognition of angiogenesis-related heparin-binding growth factors and promotion of blood vessel formation. Thus, nucleosomes constitute a novel component in tumor angiogenesis and may serve to increase cancer cell survival or tumor metastasis.

Materials and Methods

Cell Culture

HUVECs (Cambrex Co., East Rutherford, NJ) were maintained in endothelial cell basal medium (EGM-2; Cambrex) containing EGM-2 singlequot growth supplements (Cambrex). HUVECs were maintained for no more than eight culture passages.

Generation of Nucleosomes

Nucleosomes were purified from Jurkat E6-1 cells as outlined by Yager et al. (46). In brief, chromatin fragments obtained from cells swollen in hypotonic EDTA and briefly treated with *Staphylococcus aureus* micrococcal nuclease were stripped of histone H1 with carboxymethyl-Sephadex, digested

with additional micrococcal nuclease into oligomeric species, and fractionated in a Sephacryl S-300 gel filtration column. Samples containing monomeric, dimeric, or oligomeric nucleosome fractions were pooled and buffer exchanged with PBS. Purified oligomeric nucleosome stocks containing 2.6 mg of nucleohistone protein per milliliter of PBS were sterile filtered and stored at −80°C. Individual histones and nucleohistones were also obtained from Roche Biochemicals (Laval, Quebec, Canada) and Worthington Biochemicals Corp. (Lakewood, NJ), respectively. Relative nucleosome chain lengths and protein purity were determined by DNA agarose gel electrophoresis and SDS-PAGE (Fig. 6). Protein gels were stained with Bio-Rad Blue stain (Bio-Rad Laboratories, Hercules, CA), the sensitivity of which is reported to be 8-29 ng per band. Nucleosome protein and DNA concentrations also were measured colorimetrically using bicinchoninic acid (Pierce Chemical Co., Rockford, IL) and by absorbance at 260 nm, respectively.

Nucleosome Ligand Binding Assay

rFGF-1 (Invitrogen Canada, Inc., Mississauga, Ontario, Canada), rFGF-2 (Invitrogen Canada), $rVEGF_{165}$ (R&D Systems, Inc., Minneapolis, MN), human rTGF-β1 (Invitrogen Canada), human insulin growth factor-I (Cambrex), human epidermal growth factor (Cambrex), BSA (Sigma-Aldrich, St. Louis, MO), or heparin-coupled BSA (Sigma-Aldrich) were plated in triplicate at 10 μg/mL PBS into 96-well ELISA plates and incubated overnight at 4°C. Plates were washed with PBS and blocked with a PBS-2% BSA solution. Nucleosomes were suspended in the blocking solution at 0.5 μg/mL and incubated in coated plate wells at room temperature for 2 hours followed by a PBS wash. Bound nucleosomes were detected with the anti-nucleosome monoclonal antibody (10 μg/mL MAB3037, Chemicon, Temecula, CA) in conjunction with biotin-labeled goat anti-mouse antibody (Invitrogen Canada), horseradish peroxidase-coupled streptavidin (Invitrogen Canada), and 3,3′,5,5′-tetramethyl benzidine (Sigma-Aldrich). Horseradish peroxidase enzyme reactions were stopped by addition of an equal volume of 2 N H_2SO_4. Plates were read spectrometrically at 450 nm. MAB3037 was previously shown to recognize only intact nucleosomes and not individual histones, DNA, common anti-nuclear antibody targets (Ro/SS-A, La/SS-B, and Sm), basic proteins, heparan sulfate, or heparin when tested by ELISA (47).

Heparin competition for nucleosome binding to FGF-2 was performed as stated above, with the exception that 1 μg/mL heparin sulfate (Cambrex) was added to FGF-2-coated wells for 1 hour prior to the addition of nucleosomes and during the incubation of nucleosomes with FGF-2. Plates were assayed for bound nucleosomes as outlined above.

To confirm the ELISA findings for the ELISA protocol outlined above and to determine whether the nucleosome preparations were free of endogenous FGF-2, VEGF, and TGF-β1, 10 μg/mL of purified nucleosomes in PBS were initially plated in 96-well ELISA plates and incubated overnight at 4°C followed by washing with PBS and blocking with PBS-2% BSA. Nucleosome-coated wells were incubated for 1 hour at 37°C with 0.5 μg/mL rFGF-2, $rVEGF_{165}$, or rTGF-β1, washed, and incubated for 45 minutes at room temperature with 0.25 μg/mL anti-FGF-2 (BD Biosciences, San Jose, CA) or 0.5 μg/mL anti-VEGF or anti-TGF-β1 monoclonal antibodies (R&D Systems) and followed by a final wash. Plates were developed with biotin-labeled goat anti-mouse antibody and horseradish peroxidase-coupled streptavidin as outlined above. Plate wells coated with BSA or purified growth factor served as antibody background and positive well controls, respectively.

Cell Growth Assay

HUVECs previously seeded at 1×10^3 or 5×10^3 cells per well in 96-well and 24-well plates, respectively, and cultured overnight in complete culture medium were washed with serum-free medium and starved for 5 hours in 200 or 500 μL supplement-free EGM-2 medium or Opti-MEM (Invitrogen Canada) prior to the addition of 26 μg/mL nucleosome, a growth-limiting concentration of FGF-2 (20 ng/mL; ref. 48),[3] or singlequot growth medium supplements. Cells seeded in 96-well plates were cultured for an additional 36 hours; during the final 4 to 12 hours of culture, 1/10 volume of phenazine methosulfate and 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (Promega, Madison, WI) were added to measure cell metabolism by spectroscopy. Cells plated in 24-well plates were cultured for an additional 48 hours and then counted following cell trypsinization and staining with trypan blue.

Cell Migration Assay

HUVEC migration assays were carried out in Matrigel-coated Boyden chambers (BD Biosciences, Bedford, MA). Cells were harvested using versine, washed, and resuspended in supplement-free EGM-2 medium. Bottom chambers were filled with supplement-free EGM-2 medium containing 26 μg/mL nucleosome, 10 ng/mL rFGF-2, or complete EGM-2 medium containing a cocktail of angiogenic factors found in the singlequot supplement. Cells were added to the upper chamber at a concentration of 5×10^4 cells per well and cultured for 4 to 5 hours at 37°C. Adherent cells contained on the underside of the upper chamber filter were photographed in situ and counted.

In vitro Vascular Tube Formation

In vitro tube formation assays were performed as described by Malinda et al. (27). Briefly, 5×10^4 HUVECs in supplement-free EGM-2 medium were added to each well of a 24-well plate previously coated with 300 μL Matrigel (BD Biosciences). Combinations of nucleosomes and FGF-2 were added to each well; after 18 hours of culture, cells were photographed in situ. The degree of tube formation was assessed as the percentage of cell surface area versus total surface area.

In vivo Matrigel Plug Angiogenesis Assay

In vivo Matrigel plug angiogenesis assay was performed as outlined by Salcedo et al. (30). In brief, 0.4 mL Matrigel (9 mg/mL) or Matrigel mixed with 25 units/mL heparin (Sigma-Aldrich), 50 μg/mL nucleosome, or 100 ng/mL FGF-2 were injected s.c. into the midline ventral region of athymic

[3]J. Tanner, unpublished observation.

nu/nu mice (Harlan-Sprague-Dawley, St. Constance, Quebec, Canada). On day 10 postinjection, mice were sacrificed, Matrigel plugs were removed and photographed, and the level of blood vessel formation was assessed using a surrogate marker (i.e., RBC hemoglobin; refs. 31, 32). The hemoglobin assay was performed following Matrigel plug maceration with an equal volume of RBC lysis solution (Sigma-Aldrich) and overnight incubation on ice. Hemoglobin-containing samples were added to Drabkin's solution (Sigma-Aldrich) containing 0.3% Brij-35 and incubated for 30 minutes at room temperature. The hemoglobin concentration in the sample was then read spectrometrically at 540 nm and compared with hemoglobin standards (Sigma-Aldrich).

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