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Research paper

Suppression of tumorigenicity of rhabdoid tumor derived G401 cells by the multivalent HB-19 pseudopeptide that targets surface nucleolin

Bernard Krust, Diala El Khoury, Calaiselvy Soundaramourty, Isabelle Nondier, Ara G. Hovanessian*

CNRS-Université Paris Descartes, Unité Régulation de la Transcription de Maladies Génétique, 45 rue des Saints Pères, 75270 Paris Cedex 06, France

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ABSTRACT

Several studies have indicated that the cell-surface expressed nucleolin is implicated in tumorigenesis and angiogenesis, and represents an important target for cancer therapy. Here we show that treatment of rhabdoid tumor derived G401 cells with a nucleolin antagonist, the HB-19 pseudopeptide, could restore contact inhibition, impair anchorage-independent growth, and suppress tumor development in nude mice.

G401 cells grow without contact inhibition, which is an *in vitro* characteristic property of malignant tumor cells. At concentrations of HB-19 that does not affect cell viability and multiplication index, there is restoration of contact inhibition thus suggesting that HB-19 treatment causes reversion of the malignant phenotype. Accordingly, HB-19 pretreated G401 cells lose the capacity to form colonies in soft agar. When assayed for tumorigenicity in nude mice, only 50% of mice injected with HB-19 pretreated G401 cells developed tumors with the mean tumor weight of 0.32 g, compared to 100% of mice injected with control G401 cells with the mean tumor weight of 2.36 g. Interestingly, the restoration of contact inhibition in HB-19 treated G401 cells is concomitant with marked reduction of transcripts coding the Wilms' tumor 1 gene, matrix metalloproteinase-2, epithelial isoform of CD44, and vascular endothelial growth factor, whereas no apparent modification is detected for transcripts coding the proto-oncogene *c-Myc*, anti-apoptotic Bcl-2, pro-apoptotic Bax, tissue inhibitor of metalloproteinase TIMP-1, angiogenesis inhibitor TSP-1, and growth factor Midkine.

These findings indicate that the molecular mechanism of action of HB-19 on such highly malignant rhabdoid tumor cells is associated with a selective inhibitory effect on the expression of genes implicated in tumorigenesis and angiogenesis.

1. Introduction

Nucleolin is an abundant DNA-, RNA- and protein-binding protein ubiquitously expressed in exponentially growing eukaryotic cells. It is involved in fundamental aspects of transcription, cell proliferation and growth [1,2]. Nucleolin is found at several locations in cells: in the nucleolus it controls many aspects of DNA and RNA metabolism [3]; in the cytoplasm it shuttles proteins into the nucleus and provides a post-transcriptional regulation of strategic mRNAs [4,5]; and on the cell-surface it serves as an attachment protein for several ligands from growth factors to microorganisms [6–12]. In contrast to nuclear nucleolin, surface nucleolin is a constantly induced protein in proliferating tumor and endothelial cells. The half-life of surface nucleolin is less than 1 h while the half-life of nuclear nucleolin is more than 10 h [13]. Surface nucleolin is differentiated from nuclear nucleolin by a slight shift in its isoelectric point, which could reflect glycosylation of surface nucleolin [6,14,15].

Surface nucleolin serves as a low affinity receptor for HIV-1 and various growth factors that interact with its C-terminal domain containing nine repeats of the tripeptide arginine-glycine-glycine, known as the RGG or GAR domain [10,16–20]. Binding of these ligands results in clustering of cell-surface nucleolin in lipid raft membrane microdomains before endocytosis of the ligand–nucleolin complex [10,17,19]. Accordingly, surface nucleolin could shuttle ligands between the cell-surface and the nucleus thus

Abbreviations: HB-19, the surface nucleolin antagonist-pseudopeptide that presents pentavalently the tripeptide Kψ(CH_2N)PR; WT1, Wilms' tumor 1; WT1-R, WT1 related; MK, Midkine; CD44s, standard CD44; CD44E, an isoform of CD44 expressed preferentially in epithelial cells and containing the variable exons V8-V10; VEGF, vascular endothelial growth factor; TSP-1, thrombospondin-1; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* Corresponding author. Tel.: +33 0 1 4286 4136; fax: +33 0 14286 2042.
E-mail address: ara.hovanessian@parisdescartes.fr (A.G. Hovanessian).

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act as a mediator for the extracellular regulation of nuclear events [18,20,21]. Following interaction with various ligands, surface nucleolin could directly or indirectly be involved in signal transduction events [22]. For example, surface nucleolin has been shown to serve as the binding partner for receptor protein tyrosine phosphatase-sigma ectodomain in skeletal muscle [23], whereas the interaction of surface nucleolin with ErbB receptor tyrosine kinases induces receptor dimerization, phosphorylation and anchorage independent growth [24]. The binding of P-selectin to human colon carcinoma cells is shown to induce tyrosine phosphorylation of surface nucleolin and formation of a signaling complex containing nucleolin, phosphatidylinositol 3-kinase (PI3-K) and p38 MAPK [25]. Finally, ligand binding to surface nucleolin could generate high transitory intracellular Ca^{2+} membrane fluxes involving SOC-like channels, and thus initiate signaling events [14]. Consistent with this, plasma membrane localized nucleolin is shown to interact with K-Ras and play a critical role in signal transduction via the MAPK pathway [26].

The importance of cell-surface nucleolin in cancer biology was recently highlighted by studies showing that ligands of nucleolin play critical role in tumorigenesis and angiogenesis [20,25,27–36]. Accordingly, we recently reported that both of these events are suppressed by targeting surface nucleolin with the HB-19 pseudopeptide, a potent antagonist that forms an irreversible complex with surface nucleolin [9,37]. By binding to the RGG domain of nucleolin, HB-19 prevents binding of growth factors to cells, triggers calcium entry into cells, inhibits MAP kinase activation, and down-regulates surface nucleolin without affecting nuclear nucleolin [7,9,14,16,18,19,37]. In nude mice, we showed that HB-19 treatment markedly suppresses the progression of established human breast tumor cell xenografts, and in some cases eliminates measurable tumors [37]. This potent antitumoral effect *in vivo* is attributed to the direct dual inhibitory action of HB-19 on tumor and endothelial cells [37]. In a more relevant tumor model, we showed that HB-19 treatment for several months delays significantly the onset and frequency of spontaneous melanoma in RET mice, impairs tumor angiogenesis, and reduces metastasis while displaying no toxicity to normal tissue [38]. Previously, guanosine-rich quadruplex-forming oligodeoxynucleotides (GROs) that interact with surface nucleolin and/or intracellular nucleolin have been shown to be promising agents for treatment of cancer [39–41]. The aptamer AS1411 is the most recent GRO that is currently being tested in Phase II clinical trials. The proposed model for AS1411 mechanism of action involves inhibition of molecular interactions between nucleolin and several partners, such as NF-κB essential modulator NEMO, arginine methyltransferase 5 and the instability AU-rich element of *bcl-2* mRNA [42–44]. These various reports on the implication of surface nucleolin in growth of tumor cells and angiogenesis validate surface nucleolin as a strategic target for cancer therapy.

Consistent with our previous findings on breast cancer and melanoma cells [37,38], here we provide evidence to show that HB-19 has the capacity to inhibit tumorigenicity of G401 cells that are derived from a rhabdoid tumor of the kidney, one of the most aggressive and treatment-resistant pediatric cancers with very poor prognosis [45]. Our results show that HB-19 treatment has profound inhibitory effects on several critical criteria that determine tumorigenicity of such highly malignant tumor cells. Interestingly, the inhibitory effect is associated with a selective down regulation of tumor-associated genes, thus providing new insights on molecular mechanism(s) that might be involved. As current cancer drugs are rarely effective for rhabdoid tumors, there is a strong need to develop novel effective therapeutic agents [46]. Consequently, our results suggest that HB-19 is a potential candidate to consider for targeted therapy of rhabdoid tumors.

2. Materials and methods

2.1. Cells

The rhabdoid tumor cell line G401 was purchased from ATCC. This cell line that originally was reported to derive from Wilms' tumor (of a 3-month-old boy) is in fact derived from a rhabdoid tumor of the kidney [47]. G401 cells were cultured in McCoy's 5a Medium (ATCC) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.2. HB-19

The HB-19 pseudopeptide 5[Kψ(CH_2N)PR]-TASP, for [Lysψ(CH_2N)Pro-Arg]-template-assembled synthetic peptide, presents pentavalently the tripeptide Kψ(CH_2N)PR where (CH_2N) represents a reduced peptide bond between lysine and proline residues. HB-19 was synthesized by Jean Paul Briand (IBMC, Strasbourg) as described previously using solid phase peptide methodology [16,48].

2.3. Tumor cell inoculation in nude mice

All *in vivo* experiments were carried out with ethical committee approval and under the conditions established by the European Community. 8-week old female athymic nude Mice (Charles River Laboratories, France) were injected subcutaneously into the back with 4.0×10^6 control and HB-19 treated G401 cells in 0.2 ml of modified McCoy's 5a medium. Tumor development was monitored every 2–3 days and measurable tumor volume was assessed along two major axes with calipers. Tumor volume (mm^3) was calculated as follows: $V = 4/3 \times \pi \times R_1^2 \times R_2$, where R_1 is radius 1, R_2 is radius 2 and $R_1 < R_2$ [37]. All mice were sacrificed at day 50 to measure the weight of the excised tumors.

2.4. Immunofluorescence and confocal microscopy

G401 cells were plated in eight-well glass slides (Lab-Tek Brand; Nalge Nunc International, Naperville, IL) 48 h before analysis. Cells were fixed with paraformaldehyde (PFA; 3.7%, 10 min), permeabilized by Triton X-100 (0.5%, 15 min) and stained for the intracellular actin cytoskeleton using FITC-conjugated phalloidin (Sigma). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). In some experiments, nuclear nucleolin was stained using anti-nucleolin monoclonal antibody mAb D3 and Texas Red dye-conjugated horse anti-mouse IgG (from Valbiotech, Paris) [6].

2.5. Colony formation in soft agar

G401 cells (3×10^4) were mixed in 0.35% top agar diluted in McCoy's 5a Medium containing 10% FCS before plating onto 0.8% bottom agar in 12-multiwell plates. After 10–15 days, colonies with diameters superior to 50 μm were scored as positive using a phase contrast microscope equipped with a measuring grid at magnification 100×. The number of colonies was determined by analyzing 5 fields/well from 3 wells [38].

2.6. mRNA expression monitored by RT-PCR

Total RNA was prepared from G401 cells (5×10^5) using Trysol (Life technologies, Inc.) according to the manufacturer's instructions. RT was carried out with oligo(dT) and 2 μg of total RNA using Superscript II RNase H- Reverse Transcriptase (Gibco BRL). The expression of specific mRNAs was investigated by RT-PCR using primers for WT1, c-Myc, Midkine (MK), Bcl-2, Bax, VEGF,