The NK-1 receptor is expressed in human melanoma and is involved in the antitumor action of the NK-1 receptor antagonist aprepitant on melanoma cell lines

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Melanoma, the most deadly form of skin cancer, is aggressive and resistant to current therapies. It has been previously reported that the substance P and neurokinin-1 (NK-1) receptor antagonists induce cell proliferation and cell inhibition, respectively, in human melanoma cell lines. Aprepitant is a selective high-affinity antagonist of the human NK-1 receptor. Until now, this drug has been used as an anxiolytic, antidepressant and antiemetic. Moreover, the antitumor action of aprepitant has been previously reported. However, the presence of NK-1 receptors in human melanomas and whether the antitumor action of the NK-1 receptor antagonist aprepitant is exerted on human malignant melanomas have not been previously described. The aims of this study are to show the presence of NK-1 receptors in human malignant melanomas and the antitumoral action of aprepitant against several human melanoma cell lines. Immunoblot analysis was used to determine the presence of NK-1 receptors in human melanoma cell lines, and immunohistochemistry was used to demonstrate NK-1 receptors in human melanoma samples. We performed an *in vitro* study of the cytotoxicity of the NK-1 receptor antagonist aprepitant on human melanoma cell lines. A coulter counter was used to determine viable cell numbers, followed by application of the tetrazolium compound MTS. The DAPI method was applied to demonstrate apoptosis. We observed that NK-1 receptors were present in all the melanoma samples studied as well as in human melanoma cell lines. We also showed that melanoma cell lines expressed mRNA for the NK-1 receptor. Moreover, after using a knockdown method, we showed that NK-1 receptors are involved in the viability of tumor cells. In this study, we also report that aprepitant, at 10–60 μ M concentrations, elicits cell growth inhibition in a concentration-dependent manner in all melanoma cell lines studied, that the specific antitumor action of aprepitant occurs through the NK-1 receptor and that melanoma cell death is due to apoptosis. These findings show for the first time that the NK-1 receptor may be a promising new target and that the NK-1 receptor antagonist aprepitant could be a candidate as a new antitumor drug in the treatment of human melanoma.

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Melanoma is a neoplasm originating from melanocytes, which are derived from the neural crest. Melanoma now accounts for ~4% of all cancers diagnosed in the United States, and there were ~8110 deaths from the disease in 2007.¹ The last two decades have seen no significant progress in extending the survival of patients with distant metastases, despite multiple trials of cytotoxic chemotherapeutic agents.

There is thus an urgent need to improve therapy in patients with melanoma.

Substance P (SP) is an undecapeptide that belongs to the tachykinin family of peptides. It is known that SP, neurokinin A (NKA), neurokinin B and neurokinin γ (the latter two being elongated forms of NKA) are derived from the preprotachykinin A gene, whereas neurokinin B (NKB) is

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derived from the preprotachykinin B gene. The biological actions of SP, NKA and NKB are mediated by three receptors, namely neurokinin (NK)-1, NK-2 and NK-3, the NK-1 receptor showing preferential affinity for SP. After binding to the NK-1 receptor, SP regulates many biological functions.² This neuropeptide has been implicated in neurogenic inflammation, pain and depression,³ as well as in tumor cell proliferation, neoangiogenesis and metastasis.⁴ In addition, the expression of SP has been reported in primary invasive malignant melanomas, metastatic melanomas, *in situ* melanomas, atypical (dysplastic) nevi, as well as spindle and epithelioid cell (Spitz) nevi, but was not detected in any acquired benign melanocytic nevi.⁵ It has also been shown that activation of NK-1 receptors by SP induces mitogenesis in several melanoma cell lines^{6,7} and others tumor cell lines.^{8–16} SP is also a main mediator in the growth of capillary vessels *in vivo* and in the proliferation of cultured endothelial cells *in vitro*, and it has also been shown that NK-1 receptor agonists induced neoangiogenesis.¹⁷ Moreover, the active migration of tumor cells, a crucial requirement for invasion and metastasis, is regulated by SP signals.¹⁸

NK-1 receptors have been localized and characterized in several human neoplasms.^{4,7,16,19–24} Moreover, the expression of NK-1 receptors has also been reported in human glioma, neuroblastoma, pancreatic carcinoma, retinoblastoma, laryngeal carcinoma, as well as in gastric and colon carcinoma cell lines.^{10,12,15,16,21,24}

The NK-1 receptor antagonist L-733060 (a piperidine derivative) shows a high affinity for the human NK-1 receptor *in vitro*.²⁵ Moreover, we have previously shown that L-733060 shows antitumor activity against human malignant melanoma cell lines⁶ and neuroblastoma, glioma, retinoblastoma, pancreas, larynx, gastric and colon carcinoma cell lines.^{11,12,15,16,26} Aprepitant is a selective high-affinity antagonist of the human NK-1 receptor. Until now, this drug has been used as an antidepressant, anxiolytic and antiemetic.^{27–29} Despite this, we have recently reported that aprepitant is a broad-spectrum antitumor drug.³⁰ However, to our knowledge, the presence of NK-1 receptors in melanomas and whether the antitumor action of the NK-1 receptor antagonist aprepitant is exerted on human malignant melanomas are unknown. Thus, the aims of this study are: (1) to demonstrate *in vitro* the presence of NK-1 receptors in MEL HO, COLO 858 and COLO 679 human melanoma cell lines; (2) to demonstrate the presence and distribution of the NK-1 receptor in several human primary invasive malignant melanomas and metastatic melanomas; (3) to demonstrate that melanoma cell lines express mRNA for the NK-1 receptor; (4) to evaluate the involvement of NK-1 receptors on the viability of tumor cells; (5) to demonstrate, using an MTS colorimetric method to evaluate cell viability, the antitumor action of the NK-1 receptor antagonist aprepitant against human melanoma cell lines, and to show that this antitumor action occurs through the NK-1 receptor; and (6) to determine whether the NK-1 receptor

antagonist aprepitant causes apoptosis in the melanoma cell lines studied.

MATERIALS AND METHODS

Cell Culture

We used COLO 858 (ICLC Interlab Cell Line Collection-CBA-Genova), MEL HO and COLO 679 human melanoma cell lines (DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen). These cell lines were incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂ according to the manufacturer's instruction.

Drug Treatments

The NK-1 receptor antagonist 5-[[[(2R, 3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl) phenyl] ethoxy]-3-(4-fluorophenyl)-4-morpholinyl]methyl]-1,2-dihydro-3 H-1,2,4-triazol-3-one (aprepitant: MW 534.43) (Merck Research Laboratories, Madrid, Spain) was dissolved in distilled water containing acetonitrile before sample treatment. To determine IC₅₀, different concentrations (10–60 μM) of aprepitant were evaluated. SP, acetate salt (Sigma-Aldrich, Madrid, Spain), was dissolved in distilled water and different concentrations (5, 10, 50, 100 and 500 nM) were used. The most mitogenic nanomolar SP concentration for each cell line was incubated for 1 h before the addition of aprepitant.

Proliferation Assays

Cell proliferation was evaluated using the tetrazolium compound 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), according to the manufacturer's instructions (CellTiter 96 Aqueous One-Solution Cell Proliferation Assay, Promega, Madison, USA). Cell numbers were quantified using a Coulter counter. The plate included blank wells (0 cells/0.1 ml), control wells (10⁴ cells/0.1 ml), control wells with acetonitrile, control wells treated with aprepitant and control wells treated with the most effective SP concentration and aprepitant. The plates were inoculated with aprepitant (10–60 μM for tumor cell lines) and were incubated for the first doubling time specific for each tumor cell line. Plates were also inoculated with the most mitogenic exogenous SP nanomolar concentration with or without the 50% μM inhibition concentration (IC₅₀) of aprepitant for their first doubling times. Moreover, plates were also inoculated with increasing concentrations of aprepitant (30, 40, 50 and 60 μM) and for each concentration of the drug, gradual increasing concentrations of SP were added (10, 50, 100 and 500 nM). For the proliferation assay, 20 μl of the MTS reagent was added to each well 90 min before reading the samples on a multiscanner microplate reader (TECAN Spectra classic, Barcelona, Spain) at 492 nm. Each experimental condition was assayed in duplicate and all experiments were performed at least three times.

Statistical Analyses

Data were expressed as means $\pm$ s.d. Statistical analyses were performed using the SPSS statistical software for Microsoft Windows, release 13.0 (Professional Statistic, Chicago, IL, USA). The homogeneity of variance was tested using the Levene test. If the variances were homogeneous, data were analyzed using the one-way ANOVA test with Bonferroni's correction for multiple comparisons. For data sets with nonhomogeneous variances, the ANOVA test with T3 Dunnett's *post hoc* analysis was applied. The criterion for significance was $P < 0.05$ for all comparisons.

DAPI Staining

To determine whether apoptosis was induced by the NK-1 receptor antagonist aprepitant, DAPI staining was performed. Briefly, after treatment with aprepitant for their approximately first doubling times or after the application of the knockdown method (see below), the cells were fixed in 4% paraformaldehyde. After a second wash in PBS, cells were incubated in DAPI solution (Sigma-Aldrich) at a dilution of 1/1000 ($= 1 \mu\text{g/ml}$) for 30 min in the dark. Cells were then observed through a fluorescence microscope (Zeiss, Oberkochen, Germany). Apoptotic cells were defined by chromatin condensation and nuclear fragmentation. We counted the number of apoptotic cells, repeating the counts on three different slides. Finally, on each slide, we counted the number of apoptotic cells located in five different sequential fields.

Western Blot Analyses

As previously reported,¹⁰ total protein was prepared from subconfluent MEL HO, COLO 858 and COLO 679 human melanoma cell cultures. Protein concentrations were determined using the protein assay kit purchased from Bio-Rad according to the manufacturer's instructions.

From each sample, 50 μg of protein was separated by electrophoresis on 10% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. Blots were incubated in blocking solution (5% nonfat milk in PBS, 0.1% Tween-20 (PBS-T)), followed by overnight incubation with an antibody against the KTMTESSSFYSNMLA conserved domain, corresponding to the C terminus of the NK-1 receptor (Product number S8305, Sigma-Aldrich) and diluted 1:4000. Membranes were then washed with PBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 2 h at room temperature (1:10 000 dilution). Antibody detection was performed with an enhanced chemiluminescence reaction (ECL western blotting detection; Amersham Life Science, UK).

PCR

RNA extraction and reverse transcription

From cultured cells, total RNA isolation was obtained after using the NucleoSpin RNA II Kit (Macherey-Nagel), allowing the purification of $\sim 5 \times 10^6$ cultured cells. Final RNA was dissolved in RNase-free water. The purity and quality of the

RNA purified were also checked. Reverse transcription with elimination of genomic DNA was carried out according to the manufacture's instructions (QuantiTect Reverse transcription Handbook, Qiagen). We carried out all the reactions on ice to minimize the risk of RNA degradation. The cDNA obtained was kept at -80°C .

Amplification

From the cDNA preparation, 4 μl was used in PCR with specific primers (according to the modified method of Bigioni *et al*³¹ based on the common sequence of the *TAC1* human isoforms (NM 001058, NM 015727) *TAC1-F* (CTGCTGGTGATTGGCTATGC) and *TAC1-R* (AGGAGGAAGAAGATGTGGAAGG), which yielded a 186bp fragment. The amplification of the specimen was performed in a final reaction volume of 20 μl , and was incubated at 95°C for 7 min, subjected to 40 cycles of 95°C for 30 s, 62°C for 40 s and 72°C 30 s, followed by a final extension cycle at 72°C for 7 min. The amplification products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide.

Immunohistochemistry for the NK-1 Receptor

Patients

In all, 37 human malignant melanoma specimens were retrieved from the archives of the Department of Pathology of the Virgen del Rocío University Hospital (Sevilla, Spain). Of these, 27 were primary invasive melanomas of the skin and 10 were metastatic melanomas. All melanomas had at least one focus of level IV invasion according to the Clark system.³² The thickness of the tumor was evaluated according to the Breslow system.³³ The experimental design, protocols and procedures of this study were performed under the guidelines of the ethics and legal recommendations of Spanish and European law, as well as in accordance with the Declaration of Helsinki.

Methods

Paraffin-embedded human malignant melanoma tissues cut at 5 μm and dried overnight at 60°C were used. As previously reported by us,¹⁶ to determine the specificity of the immunostaining, human gliomas were used as a positive control, whereas as a negative control the primary antibody was omitted, being replaced by nonimmune serum, and in both cases, the results obtained confirmed the specificity of the anti-NK-1 receptor antibody (Product number S8305, Sigma-Aldrich) used. Moreover, the anti-NK-1 receptor was preabsorbed (100 μg per ml of diluted antiserum) with the synthetic peptide KTMTESSSFYSNMLA (synthesized by GenWay Biotech, San Diego, USA; see western blot analyses) and after the immunocytochemical protocol, no immunostaining appeared. All slides were evaluated by three independent pathologists. In all, 10 representative high-power microscopic fields were evaluated on each slide using a $\times 40$ objective. The presence or absence of staining and the

intensity of the immunoreactivity were noted, as well as the number of cells showing a brown staining and whether the staining was localized in the tumor cells and/or in the plasma membranes. Tumors were recorded as positive when they showed cellular and/or plasma membrane staining ranging from moderate to strong in >10% of the tumor cells. By consensus among the pathologists, the intensity of the immunoreactivity was evaluated as 'acceptable' only if the tumor cells showed a clear brown staining localized in the cytoplasm and/or plasma membrane of the tumor cells. The number of 'acceptable' immunoreactive cells was quantified as follows: negative (0) if the immunoreactivity was observed in <10% of the tumor cells; positive (+) if the immunoreactivity was found to be between 11 and 50% of the tumor cells; and very positive (++) if it was present in >50% of the tumor cells (see Table 1).

Small Interfering RNA Gene Silencing Method

We carried out this method according to the manufacturer's instructions (Invitrogen, Madrid, Spain) and according to a previous study.³⁴ One day before transfection, 2×10^4 MEL HO human melanoma cells per well were seeded in 6-well plates containing 2 ml of normal growth medium. Total cell number was determined the day before the transfection. Cells were incubated at 37°C in a CO₂ incubator for 17–20 h until 50–60% of the cells were confluent. The normal growth medium was then removed, and an antibiotic-free growth medium (Opti-MEM; Gibco) was added and incubated for 1 h. The latter medium was removed and the transfection mixture was added, which contained the small interfering RNA (siRNA) TAC 1 (Invitrogen) and the diluted transfection reagent medium (Hiperfect, Qiagen); the two latter were previously incubated for 30 min. Thereafter, the final volume obtained was 200 μ l. In the same manner it was applied to siRNA-negative control solution. For each transfection, 200 μ l siRNA transfection reagent mixture was added to each well containing 800 μ l of the antibiotic-free growth medium. In all, 20 nM was the final siRNA concentration used for each transfection. It was incubated for 5 h at 37°C in a CO₂ incubator. Finally, 2 ml of normal growth medium was added for additional 48–72 h incubation.

RESULTS

NK-1 Receptors

We carried out western blot analyses to test the presence of NK-1 receptors in MEL HO, COLO 858 and COLO 679 human melanoma cell lines. Incubation of the membranes with the anti-NK-1 receptor antibody (see the 'Materials and methods' section) showed the presence of different isoforms of the NK-1 receptor in human melanoma cell lines studied (Figure 1). Three similar bands (isoforms of ~33, 58 and 75 kDa) were observed in the three melanoma cell lines studied (Figure 1b).

As a positive control, we included a protein extract from a glioma cell line. As previously reported¹⁰, we observed bands

of 33, 37 and 75 kDa (see Figure 1c). In addition, no band was detected when the incubation was carried out with the secondary antibody alone (see Figure 1a).

By PCR analyses, we also showed that MEL-HO, COLO 858 and COLO 679 tumor cell lines expressed mRNA for the tachykinin NK-1 receptor (Figure 2). NK-1 mRNA expression was detectable in these cell lines as a product of the expected size of 186 bp corresponding to only one band, because the primers used in our PCR were designed for both short and long isoforms.

The knockdown method was carried out in two of the three melanoma cell lines studied. Thus, after 72 h, we found 4×10^5 siRNA-negative control MEL HO cells and 1×10^5 siRNA TAC 1 cells (Figure 3a). Moreover, after the administration of siRNA TAC 1 to MEL HO cultured cells, many apoptotic cells were found in all the wells studied. In fact, in DAPI-stained cultures, we observed means of 40 ± 1.4 (s.d.)% of apoptotic cells. However, in siRNA-negative control MEL-HO cells, we observed means of 5 ± 0.4 (s.d.)%. With regard to COLO 679 melanoma cells, after 72 h, we found 7.2×10^5 siRNA-negative control cells and 1.8×10^5 siRNA TAC 1 cells (Figure 3b). As before, after the administration of siRNA TAC 1 to COLO 679 cultured cells, many apoptotic cells were found. Thus, in DAPI-stained cultures, we observed means of 34 ± 2.7 (s.d.)% of apoptotic cells, but in siRNA-negative control COLO 679 cells, we observed means of 6 ± 0.5 (s.d.)%. All these data show that NK-1 receptors have an important role in the viability of such tumor cells.

NK-1 Receptors in Primary Invasive Malignant Melanoma of the Skin and Metastatic Melanoma

Using an immunohistochemical technique, 37 human malignant melanomas were examined for the presence of NK-1 receptors. The expression of NK-1 receptors was observed in all the malignant melanoma samples studied (37/37), in both primary invasive malignant melanomas (27/27) and metastatic melanomas (10/10). Immunoreactivity was predominantly located in the cytoplasm of tumor cells (see Figures 4c and 5). Moreover, in three cases,^{19,20,26} an enhancement of the staining in the plasma membrane of these tumor cells was observed (Figure 4c). In seven of the primary invasive malignant skin melanomas (see Table 1), immunoreactivity for NK-1 receptors was observed in the smooth muscle cells of the small- and medium-caliber blood vessels, which were located in the peritumoral area, and occasionally, such immunoreactivity was also observed in the endothelial cells of these vessels. In 11 cases (see Table 1), immunoreactivity was observed in hair follicles, sebaceous glands or in sweat glands and in 17 of them, keratinocytes showed NK-1 immunoreactivity (see Table 1). Both follicles and glands showed cytoplasmic immunoreactivity, even in cases in which such structures were inside the tumor. Keratinocytes of the thorny and granular strata of the supratumoral epidermis showed an evident nuclear

Table 1 Clinical findings: evaluation of the expression of the NK-1 receptor and its immunolocalization in tumor cells of primary invasive malignant melanomas of the skin and metastatic melanomas (M)

Patients	Sex	Age (years)	Location	Breslow system (mm)	Immunohistochemical expression of NK-1 receptor	Immunoreactivity location in tumor cells	Immunoreactivity in keratinocytes stratum spinosum/basale/granulosum	Immunoreactivity in smooth muscle cells, endothelial cells of blood vessels	Immunoreactivity in hair follicles, sebaceous glands sweat glands
1	Man	57	Back	2	++	Cytoplasm	0	0	0
2	Woman	44	Pubis	15	++	Cytoplasm	0	0	+
3	Woman	41	Back	1.8	+	Cytoplasm	0	0	0
4	Man	36	Shoulder	2.71	+	Cytoplasm	0	0	0
5	Woman	37	Skin of breast	2.05	+	Cytoplasm	+	+	0
6	Man	54	Cheek	6.4	++	Cytoplasm	+	+	+
7	Woman	51	Back	1.6	++	Cytoplasm	+	0	+
8	Man	60	Leg	4.6	++	Cytoplasm	+	+	0
9	Woman	48	Shoulder	2.2	+	Cytoplasm	0	0	0
10	Man	60	Neck	9.2	++	Cytoplasm	+	0	+
11	Woman	60	Vulva	1.95	+	Cytoplasm	0	0	0
12	Man	69	Back of foot	6.5	++	Cytoplasm	0	0	0
13	Man	42	Back	3.2	++	Cytoplasm	+	0	+
14	Woman	69	Sole of foot	2.45	++	Cytoplasm	+	+	+
15	Man	69	Lumbar region	6.2	+	Cytoplasm	0	0	0
16	Woman	37	Thigh	1.35	++	Cytoplasm	+	+	+
17	Man	38	Toe	14	++	Cytoplasm	+	+	0
18	Woman	61	Scalp	13	++	Cytoplasm	+	0	0
19	Man	35	Leg	6	++	Cytoplasm/ membrane	+	0	0
20	Man	49	Scalp	20	++	Cytoplasm/ membrane	0	0	+
21	Woman	56	Leg	6.7	+	Cytoplasm	+	0	0
22	Man	58	Abdomen	3	++	Cytoplasm	+	+	0
23	Woman	57	Back	3.9	++	Cytoplasm	+	0	+
24	Man	38	Scalp	3	+	Cytoplasm	+	0	+
25	Woman	66	Vulva	6.2	+	Cytoplasm	+	0	0
26	Man	60	Back	5.5	++	Cytoplasm/ membrane	0	0	0
27	Woman	28	Leg	2.3	++	Cytoplasm	+	0	+
M1	Woman	59	M Soft tissues		++	Cytoplasm			
M2	Woman	74	M Bone		++	Cytoplasm			
M3	Woman	33	M Brain		+	Cytoplasm			
M4	Woman	58	M Lymph nodes		++	Cytoplasm			
M5	Man	54	M Lymph nodes		+	Cytoplasm			
M6	Man	74	M Soft tissues		++	Cytoplasm			
M7	Man	59	M Lymph nodes		++	Cytoplasm			
M8	Man	40	M Pleura		++	Cytoplasm			
M9	Man	33	M Lymph nodes		+	Cytoplasm			
M10	Woman	46	M Pleura		++	Cytoplasm			

The expression of the NK-1 receptors is also evaluated in keratinocytes, smooth muscle cells, hair follicles and in sebaceous and sweat glands.

Figure 1 Western blot analysis of the NK-1 receptor in MEL HO, COLO 858 and COLO 679 human melanoma cell lines, showing the presence of different NK-1 receptor complex isoforms (**b**). The analysis was conducted three times. Dots indicate bands with molecular weights similar to those reported previously. (**a**) No band was detected when incubation was carried out with the secondary antibody alone. (**c**) Positive control: a protein extract from a glioma cell line (GAMG).¹⁰ In this cell line, bands of 33, 37 and 75 kDa were observed.

Figure 2 Agarose gel showing expression of *TAC1-R* cDNA in human melanoma cell lines. Equal aliquots of each cDNA were amplified by PCR for 40 cycles (target genes) with specific primers. PCR was carried out three times.

immunoreactivity. This suggests that the interior of the nucleus might have NK-1 receptors. In six cases of primary invasive malignant melanomas, the peritumoral area was not evaluated because the sample contained only tumor. The clinical data of the patients included in this study, together with the results of the evaluation of the expression of NK-1 receptors in tumor and no tumor cells and the location of such expression, are shown in Table 1 (also see Figures 4 and 5). Thus, for example, the expression of the NK-1 receptors was reported as positive (between 11 and 50% of the tumor cells showed immunoreactivity) and very positive (>50%). This means that we observed immunoreactive and nonimmunoreactive cells in the same histological section (see Figure 5g, h).

Antitumor Action of Aprepitant

Growth inhibition of melanoma cell lines by aprepitant was observed after the addition of increasing concentrations of the drug (Figure 6a). Moreover, treatment of the three tumor cell lines with aprepitant resulted in concentration-dependent cytotoxicity (see Figure 6a). The IC₅₀ and IC₁₀₀ growth

Figure 3 Viability of MEL HO and COLO 679 melanoma cells. This experiment was repeated three times in duplicate. The number of siRNA-negative control cells is compared with that of siRNA *TAC1* cells. At time 0, 30 000 MEL HO and 100 000 COLO 679 cells were seeded. In both cases, at 72 h, the decrease in the number of siRNA *TAC1* cells was quite significant in comparison with the number of siRNA-negative control cells.

inhibition aprepitant concentrations of melanoma cell lines are shown (in Table 2). Maximum inhibition was observed when the drug was present at a concentration of ~60 μ M during the culture periods. At the first doubling time, a strong decrease in the number of melanoma cell lines studied

Figure 4 Case 20: (a) Primary malignant skin melanoma. Hematoxylin–eosin-stained: medium-power magnification. Epidermis and dermis can be observed in the delimited region. (b) No immunoreactivity for NK-1 receptors was observed in keratinocytes of the granular and thorny strata. (c) Strong immunoreactivity for NK-1 receptors was observed in tumor cells. Medium-power magnification. An enhancement of the staining in the plasma membrane of such cells is noted. The immunocytochemical technique was repeated at least three times.

Figure 5 Case 16: (a) Primary malignant skin melanoma. Hematoxylin–eosin-stained: medium-power magnification. (b) Negative control: medium-power magnification. (c, d) Immunoreactivity located in tumor cells: medium- and high-power magnification, respectively. Case M1: (e) Metastatic soft tissue melanoma, Hematoxylin–eosin-stained: medium-power magnification. (f) Negative control: medium-power magnification. (g, h) Immunoreactivity located in tumor cells: medium- and high-power magnification, respectively. In panels g and h, the presence of nonimmunoreactive cells (arrows) must be noted. In both 16 and M1 cases, the immunocytochemical technique was repeated at least three times.

was found at intermediate concentrations and no remaining living cells were observed at the maximum concentration. In all cell lines studied, a lower inhibition of growth was observed in the presence of low doses of aprepitant. There were no significant differences between the controls and the control-acetonitrile groups (data not shown).

The NK-1 Receptor Antagonist Aprepitant Blocks SP-Induced Mitogen Stimulation

Our group has previously reported that the three melanoma cell lines studied herein grew after the addition of different doses of SP.⁶ To show that the NK-1 receptor antagonist aprepitant inhibits melanoma cell proliferation by interaction with its receptor, we used the specific NK-1 receptor agonist SP in competition experiments. Thus, the cellular con-

centration at the μM IC_{50} of aprepitant and the most mitogenic nanomolar concentration of SP were lower than those observed after using different doses of SP alone for the three melanoma cell lines studied. These results indicate that aprepitant blocks SP mitogen stimulation, and that aprepitant-induced growth inhibition was partially reversed by the administration of the most mitogenic nanomolar dose of exogenous SP. In this sense, we observed higher cellular concentrations when compared with the data found after the administration of aprepitant alone (see Figure 7). This indicates that SP specifically activates NK-1 receptors during the growth of human melanoma cell lines.

Moreover, aprepitant-induced growth inhibition was partially reversed by the administration of gradual increasing concentrations of SP. The maximum effect was observed after

Figure 6 (a) Percentage of growth inhibition of MEL HO, COLO 858 and COLO 679 human melanoma cells at first doubling time in *in vitro* cultures after the addition of increasing concentrations (10–60 μM) of aprepitant. Level of significance: $**P \leq 0.05$. The experiment was repeated three times in duplicate. (b) MEL HO human melanoma: aprepitant-induced growth inhibition was gradually reversed by the administration of gradual increasing concentrations of SP. Level of significance: $**P \leq 0.05$. The experiment was conducted three times in duplicate.

Table 2 Results of half-inhibition (IC₅₀) and maximum inhibition (IC₁₀₀) experiments in melanoma cells after administration of the NK-1 receptor antagonist aprepitant

Melanoma cell line	Aprepitant	
	IC ₅₀ μM	IC ₁₀₀ μM
MEL HO	29.6	56.5
COLO 858	24.3	52.1
COLO 679	32.1	58.6

the administration of 50 nM of exogenous SP, in competition with different concentrations of aprepitant (Figure 6b). This means that aprepitant does not exert an off-target effect on melanoma cell lines and that the antitumor action of aprepitant is mediated through NK-1 receptors.

Figure 7 Induction of cell proliferation of COLO 679 human melanoma cells by SP at several nanomolar concentrations (5, 10 and 50 nM). The experiment was conducted at least three times in duplicate. The NK-1 receptor antagonist aprepitant was added (30 μM) in the presence (10 nM) or absence (none) of SP for the first doubling time. In both cases, aprepitant inhibited COLO 679 cell proliferation. Using the ANOVA test, a significant difference between each group and the control group (none–none) was found. Level of significance: $**P \leq 0.05$ and # indicates the value of significance of 10–none vs 10–30 and × 10–30 vs none–30 # and × $P \leq 0.05$. Vertical bars indicate s.d.

Apoptosis

After the administration of aprepitant, many apoptotic cells were found in all melanoma cell lines studied (Figure 8). In fact, in DAPI-stained cultures, we observed means of 28 ± 8.1 (s.d.)% at the IC₅₀ concentration and of 36 ± 7.6 (s.d.)% at the IC₁₀₀ concentration of apoptotic cells in all melanoma cell lines studied after the administration of aprepitant. Conversely, in control melanoma cells (not treated with aprepitant), we observed means of 0.57 ± 0.6 (s.d.)% of apoptotic cells in all three melanoma cell lines.

DISCUSSION

In this study, we showed for the first time the presence of NK-1 receptors in MEL HO, COLO 858 and COLO 679 human melanoma cell lines and the presence of NK-1 receptors in human primary invasive malignant melanomas and metastatic melanomas.

We carried out several controls to check the specificity of the anti-NK-1 receptor antibody used in this study: (1) omission of the primary antibody (immunohistochemistry and western blot); (2) preabsorption of the primary antibody with the corresponding synthetic peptide (immunohistochemistry); and (3) the use of human gliomas as positive controls (immunohistochemistry and western blot). In all the cases, the results confirmed the specificity of the anti-NK-1 receptor antibody used in this study. It should also be noted that in the same human melanoma sample, we observed cells expressing or not expressing NK-1 receptors. Moreover, after eliminating NK-1 receptors by a knockdown method, we showed that NK-1 receptors are involved in the viability of tumor cells, as well as that these two melanoma cell lines express mRNA for the NK-1 receptor.

Figure 8 (a) Untreated COLO 858 melanoma cell culture. (b) COLO 858 melanoma cells treated with the NK-1 receptor antagonist aprepitant. The experiment was repeated three times. Apoptotic figures: the chromatin condensation and the nuclear fragmentation can be observed. (c) High-power magnification of the region delimited in panel b.

The data described herein are in agreement with those reported in previous studies, in which the presence of isoforms of the NK-1 receptor in neuroblastoma, glioma, retinoblastoma, pancreatic, larynx, colon and gastric carcinoma cell lines was reported.^{10,12,15,16,24} We show the presence of several NK-1 receptor isoforms (33, 58 and 75 kDa) in MEL HO, COLO 858 and COLO 679 human malignant melanoma cell lines, although the functional roles of these NK-1 receptor isoforms are currently unknown. Until now, the expression of NK-1 receptors in human primary invasive malignant melanomas and metastatic melanomas was unknown. In this study, for the first time, we show the presence of NK-1 receptors in both cases. Our data are in agreement with those of previous studies, as it is known that NK-1 receptors are overexpressed in primary glioblastomas, breast cancer and its metastasis, pancreatic carcinoma, retinoblastoma, and larynx, gastric and colon carcinomas.^{16,19–22,24} Moreover, it has been shown that NK-1 receptor expression is increased 25–36-fold in human pancreatic cancer cell lines in comparison with normal controls, and that tumors samples obtained from patients with advanced tumor stages exhibit significantly higher NK-1 receptor levels.²¹ Thus, using immunohistochemical methods, NK-1 receptor visualization should facilitate the identification of tumors with a sufficient NK-1 receptor overexpression for diagnostic and therapeutic intervention (administering NK-1 receptor antagonists).^{4,23} It is known that the activation of the NK-1 receptor by SP at nanomolar concentrations induces mitogenesis in human malignant melanoma cell lines⁶ and in several cancer cell lines,^{8,10–13,15,16} and also that SP is expressed in primary invasive malignant melanomas and metastatic melanomas.⁵

We showed that treatment of the three melanoma cell lines studied herein with the NK-1 receptor antagonist aprepitant causes growth inhibition and cell death. We also showed that the cell death observed in this study was due to a specific toxic effect of aprepitant and not a nonspecific action of this

drug. In our competition experiments, exogenous SP cell proliferation was partially reversed by the administration of aprepitant in the three melanoma cell lines, indicating the specificity of NK-1 receptor blockade by aprepitant. Moreover, aprepitant-induced growth inhibition was gradually reversed by gradual increasing concentrations of SP. Thus, the antitumor action on these melanoma cell lines by aprepitant is probably related to the ability of this antagonist to block the NK-1 receptor. In addition, the findings of this study show that treatment of the three melanoma cell lines with this NK-1 receptor antagonist results in cell death and that such death occurs by apoptosis. This is in agreement with previous *in vitro* studies carried out in lung cancer,³⁵ rhabdomyosarcoma,⁷ neuroblastoma, retinoblastoma, and larynx, gastric and colon carcinoma cell lines.^{14–16,24}

In a previous study, it has been reported that NK-1 receptors mediate shape changes in normal human embryonic kidney cells (HEK 293) and that these changes were prevented after preincubation with NK-1 receptor antagonists at 1 μ M concentration.³⁶ We have previously reported that the IC₅₀ for the NK-1 receptor antagonist aprepitant against HEK 293 cells, was >90 μ M (30). At 1 μ M of aprepitant, we did not observe any antitumoral action on the tumor cells studied herein. On the contrary, we observed such action when the concentration of aprepitant was increased. This could be explained because tumor cells overexpress NK-1 receptors,⁴ and for this reason, it is necessary to increase the concentration of NK-1 receptor antagonists to observe an antitumoral action on cancer cells. Previously, the effect of SP on the growth of capillary vessels *in vivo* and on the proliferation of cultured endothelial cells *in vitro* has been reported. In this sense, neoangiogenesis, probably due by endothelial cell proliferation, could be blocked specifically by NK-1 receptor antagonists.^{17,37} Furthermore, neoangiogenesis is a sequential process with early endothelial proliferation, followed by vessel neoformation and increased blood

flow; the maturation of endogenous neurovascular regulatory systems occurs late in this process in inflamed tissues.^{38,39} Vessel neoformation has also been associated with increased tissue innervation and expression of NK-1 receptors;²⁰ indeed, in a large majority of the tumors investigated, NK-1 receptors have been found on intratumoral and peritumoral blood vessels¹⁹ and this finding is in agreement with the results reported herein. In addition, both SP and histamine (released from mast cells by the action of SP) are often concentrated in the connective tissue surrounding many tumors, including melanomas, and become so before the development of neoangiogenesis.⁵ The findings reported in this study show that the expression of the NK-1 receptor in tumor vessels is consistent with the above studies. Conversely, SP analog antagonists, synonymous of NK-1 receptor antagonists, inhibit tumor growth in pancreatic cancer by a dual mechanism involving both antiproliferative and anti-angiogenic properties.⁴⁰ The active migration of tumor cells is a crucial requirement for the development of metastasis and cancer progression. It is known that SP induces the migration of tumor cells to specific organs by binding to NK-1 receptors in cancer cells, where it can be blocked by NK-1 receptor antagonists.¹⁸ Moreover, a recent study carried out in HEK 293 cells has reported that the activation of the NK-1 receptor by SP induced a rapid cellular shape change, including blebbing. This is not associated with apoptosis, but it is a consequence of the Rho-activated ROCK system, leading to an increase in the phosphorylation of the myosin regulatory light chain.³⁶ Membrane blebbing is important in cell movement, cell spreading and in cancer cell invasion.⁴¹ The same study reported that aprepitant and L-733060 blocked SP-induced rapid cellular shape change, including blebbing.³⁶

These data suggest that SP and NK-1 receptors could have an important role in the development of invasion and metastasis. In addition, some alarming signs of the malignization of the nevi are pain, pruritus, inflammation and increased size. These signs could be due to an increase in SP in the perilesional zone of the skin produced by the different mechanisms involved in neurogenic inflammation. In fact, the release of SP from peripheral sensory fibers could produce vasodilatation, increase vascular permeability and plasma extravasation, as well as induce the release of histamine (pruritus) from skin mast cells. In addition, melanoma cells releasing SP could cause tumor proliferation.

All the data mentioned above suggest that the use of NK-1 receptor antagonist aprepitant could improve melanoma treatment because the drug could exert an antitumoral action through three mechanisms: (1) an antiproliferative effect, due to the inhibition of tumor cell growth, inducing cell death by apoptosis; (2) an inhibition of angiogenesis in the tumor mass; and (3) an inhibition of the migration of tumor cells (invasion and metastasis). Moreover, aprepitant has also been used in a placebo-controlled trial in patients with depression, with the observation that the safety and the tolerability of this drug are generally similar to the placebo.²⁷ Recently, we have

reported the safety of aprepitant against human fibroblast cells (HEK 293). Thus, the IC₅₀ for HEK 293 fibroblast cells was approximately three times higher than the IC₅₀ for tumor cells,³⁰ as occurring in the melanoma cell lines studied here. Indeed, in the host, aprepitant exerts beneficial effects in a dose-dependent manner, such as antiemetic,²⁹ antidepressant,²⁷ anxiolytic,²⁸ anti-inflammatory,⁴² analgesic,⁴³ hepatoprotector⁴² and neuroprotector.⁴⁴

In summary, we describe for the first time the presence of several isoforms of the NK-1 receptor in MEL HO, COLO 858 and COLO 679 human malignant melanoma cell lines. We identified the presence of NK-1 receptors in all human primary invasive malignant melanomas and metastatic melanomas studied, and we report the antitumoral action of the NK-1 receptor antagonist aprepitant on human melanoma cell lines. We also show that the antitumoral action of aprepitant is through the NK-1 receptor and that aprepitant induces apoptosis in melanoma cell lines. Finally, we have also reported that NK-1 receptors have an important role in the viability of tumor cells and that the above-mentioned melanoma cell lines express mRNA for the NK-1 receptor. All these observations suggest that the NK-1 receptor is a new candidate target in the treatment of human melanoma and that the NK-1 receptor antagonist aprepitant could be a novel and promising antitumor drug in the treatment of melanoma.

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DISCLOSURE/CONFLICT OF INTEREST

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