Melanoma Research 2002, 12, pp. 1–10

Androgen receptors in human melanoma cell lines IIB-MEL-LES and IIB-MEL-IAN and in human melanoma metastases

V. Morvillo, I. A. Lüthy*, A. I. Bravo, M. I. Capurro, P. Portela, R. S. Calandra and J. Mordoh

Centro de Virología Animal (CEVAN-CONICET), Serrano 669 (1414) Buenos Aires, Argentina (V. Morvillo). Instituto de Biología y Medicina Experimental, Obligado 2490 (C1428ADN) Buenos Aires, Argentina. Fax: (+54) 11 4786 2564; Email: iluthy@ibyme.dna.uba.ar (I. A. Lüthy, R. S. Calandra). Policlínico Eva Perón, Balcarce 900 (1650) San Martín, Provincia de Buenos Aires, Argentina (A. I. Bravo). Instituto de Investigaciones Bioquímicas, Fundación Instituto Leloir, (IIB-BA, IIB-FCEYN), Patricias Argentinas 435 (C1405BWE) Buenos Aires, Argentina (M. I. Capurro, P. Portela, J. Mordoh). Cátedra Endocrinología, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, calle 47 y 115, (1900) La Plata, Provincia de Buenos Aires, Argentina (R. S. Calandra).

The presence and characteristics of androgen receptors (ARs) have been described by our group in one human melanoma cell line. We have now investigated their presence in two other human melanoma cell lines, IIB-MEL-LES and IIB-MEL-IAN, as well as in biopsies from human metastatic melanoma. Scatchard analysis revealed a single binding component for both cell lines, the apparent dissociation constant obtained being 15 nM, with a binding capacity of 280 fmol/mg total cell protein, for IIB-MEL-LES cells and 14 nM, with a binding capacity of 206 fmol/mg total cell protein for IIB-MEL-IAN cells. When specificity was assessed, not only androgen and anti-androgen but also non-androgenic compounds were able to compete for [^{3}H]R1881 binding, as seen before. When immunocytochemistry of IIB-MEL-LES and IIB-MEL-IAN cells was performed for ARs, both cell lines were deeply stained in the nucleus, whereas no staining was found for oestrogen or progesterone receptors. Every specimen of melanoma metastases tested for the presence of ARs was deeply stained, and in the majority the intensity of the staining was high. Several hormones and anti-hormones were tested for their ability to affect cell proliferation. In both cell lines, testosterone, dihydrotesterone, oestradiol and progesterone significantly stimulated cell proliferation, and this was reversed by hydroxyflutamide, bicalutamide or tamoxifen.

Keywords: androgen, androgen receptor, anti-androgen, anti-oestrogen, flutamide, melanoma, tamoxifen

*To whom correspondence should be addressed

DOI: 10.1097/01.cmr.0000043153.28051.51

Introduction

Between 1940 and 1990, epidemiological studies documented a steady rise in the incidence of cutaneous melanoma in all countries where statistics are maintained. For example, in the USA the incidence rate has been rising 4–6% each year since 1973. Melanoma-associated mortality also increased during the same period in most parts of the world [1,2]. Gender was found to be a significant prognostic factor, with Males having worse survival than Females. This is true for both early stage and advanced stage patient groups, the relative risk being 1.33 for Males [2].

A first attempt to explain this difference suggested that oestrogen receptors (ERs) could be present in human melanoma cells [3]. Several phase II trials were conducted with the anti-oestrogen tamoxifen as a single agent or combined with chemotherapy (reviewed by Rusthoven [4]), with contradictory results.

It has also been theoretically proposed [5] that an androgen dependency of melanoma could explain the longer survival time in Female patients, irrespective of the hormonal status of the Female host. It might also explain the incidental occurrence of rapid progression of melanoma during gestation and the spontaneous regressions sometimes associated with the termination of pregnancy, for abundant quanti-

Article number = 2231

ties of androgen precursors are secreted during pregnancy.

Our group has demonstrated the existence of androgen receptors (ARs) in the human melanoma cell line IIB-MEL-J using the binding of tritiated methyltrienolone and immunohistochemistry. Binding studies provided evidence of atypical specificity, with competition by androgenic and non-androgenic compounds. Most importantly, incubation of these cells in the presence of androgen significantly stimulated cell proliferation, and this stimulation could be reversed by either the anti-androgen flutamide (or its active metabolite hydroxyflutamide) or the anti-oestrogen tamoxifen. Flutamide administered to nude mice inoculated with melanoma cells was effective in diminishing tumour growth and increasing the survival rate of the animals [6].

In order to gain further information about these atypical characteristics in a melanoma cell line, a comparative study was designed with two other human melanoma cell lines. In the present study, we demonstrated a clear stimulation of cell proliferation by androgen as well as non-androgen compounds (oestradiol and progesterone). We also demonstrated an inhibitory effect of anti-androgens and anti-oestrogens on cell growth *in vitro*, and the presence of ARs with atypical sensitivity to different steroids in both cell lines. The presence of ARs was confirmed by immunohistochemistry. Moreover, all specimens from melanoma metastases tested for the presence of ARs were clearly stained, and in the majority the intensity of the staining was high.

Materials and methods

Materials

[^{3}H]17β-Hydroxy-17α-methyl-oestra-4,9,11-trien-3-one ([^{3}H]R1881) (86.5 Ci/mmol), [2,4,6,7-^{3}H]oestradiol ([^{3}H]E_2) (85 Ci/mmol), 17α-methyl-[^{3}H]promegestone ([^{3}H]R5020) (87 Ci/mmol), and radio-inert R1881 and R5020 were purchased from DuPont NEN Research Products, Boston, Massachusetts, USA.

Dulbecco's modified Eagle's medium, Ham's F12 medium, fetal calf serum (FCS) and trypsin were obtained from GIBCO, Grand Island, New York, USA. Sodium selenite, ascorbic acid, galactose, sodium pyruvate, ethylene diamine tetra-acetic acid (EDTA), diethylstilboestrol (DES), oestradiol (E_2), triamcinolone acetonide (TA), dihydrotestosterone (DHT), testosterone, cortisol, progesterone and 11-desoxycorticosterone (DOCA) were all purchased from SIGMA Chemical Co, St Louis, Missouri, USA.

Monoclonal anti-androgen receptor antibody (Clone AN1-15) was obtained from Affinity BioReagents (Golden, Colarado, USA). It was raised against fusion protein-A containing about 40% of the N-terminal domain and 25% of the DNA-binding domain of the human AR. The monoclonal anti-oestrogen receptor (ER-ICA) and monoclonal anti-progesterone receptor (PR-ICA) were kits obtained from Abbott Laboratories, North Chicago, Illinois, USA.

Melanoma cell lines

The IIB-MEL-LES cell line was originally established from an axillary lymph node metastasis from a 42 year old Male Caucasian patient, and the IIB-MEL-IAN cell line from an amelanocytic lymph node metastasis from a 41 year old Male patient [7]. These cell lines were maintained in Dulbecco's modified Eagle's medium:Ham's F12 medium (1 : 1) supplemented with 10% FCS, 2 mM glutamine, 20 nM sodium selenite, 100 μM ascorbic acid, 0.3 mg/ml galactose and 0.15 mg/ml sodium pyruvate (melanoma medium). Cells were usually plated in the same medium in 75 cm^2 flasks in a 5% CO_2 in air atmosphere at 37°C. The medium was changed twice weekly. Cells were subcultured at confluence by gentle dispersion with 0.25% trypsin and 0.065% EDTA.

Scatchard plot analysis and competition studies of androgen binding

For androgen binding, [^{3}H]R1881 incorporation into living cells was performed as already described elsewhere [8]. Briefly, IIB-MEL-LES and IIB-MEL-IAN cells were grown in 35 mm diameter Petri dishes in melanoma medium. When cells reached near-confluence status, the medium was removed, washed three times with Dulbecco's phosphate-buffered saline (PBS) and fresh medium without serum was added. After 24 h, cells were washed again three times with PBS and incubated for 2 h at 37°C in fresh medium in the presence of increasing concentrations of [^{3}H]R1881 and 1000-fold excess TA [9] and in the presence or absence of 100-fold excess unlabeled R1881. Cell numbers ranging from $0.25–0.6 \times 10^6$ cells were used in the experiments. After the 2 h incubation, cells were washed three times with PBS, trypsinized and counted in a liquid scintillation counter.

For the competition studies, cells were prepared following an identical protocol. Incubation was

performed for 2 h with 22 nM [^{3}H]R1881 for IIB-MEL-LES cells and 9 nM [^{3}H]R1881 for IIB-MEL-IAN cells, in the presence or absence of several steroids.

Immunohistochemistry of androgen, oestrogen and progesterone receptors

Immunohistochemistry for ARs [10] and for ERs and progesterone receptors (PgRs) [11] as described elsewhere. Briefly, $5-10 \times 10^6$ IIB-MEL-LES or IIB-MEL-IAN cells were trypsinized, washed three times with PBS, included in a drop of embedding medium (Tissue-Tek OCT compound, Miles Laboratories, Naperville, Illinois, USA) and frozen. Successive 3–5 μm cryostatic sections of the cell preparation were mounted on poly-L-lysine-treated slides and immediately fixed on 3.7% (for ERs and PgRs) or 2% (for ARs) formaldehyde with the usual washes. Alternatively, cells grown directly over glass slides were used and treated in the same way as cell pellets. A peroxidase–anti-peroxidase system (Abbot ER-ICA monoclonal kit and Abbot PgR monoclonal antibody) was used for the detection of ERs and PgRs, with the human breast cancer cell line MCF-7 being used as the positive control. AR determination was performed with anti-AR AN1-15 monoclonal antibody and revealed with Vectastain ABC Kit (peroxidase, Vector Laboratories, Burlingame, CA) [10]. Biopsies from patients were used in order to evaluate the presence of ARs in tumour tissues, whereas normal tissues were from obtained from autopsy specimens. Tumour tissues were separated from fat and necrotic or normal tissue, cut into 1 mm^3 fragments and embedded in optimal cutting temperature (OCT, from Sakura Finetek USA, Torrance, CA).

Cell growth experiments

For the cell growth experiments, IIB-MEL-LES and IIB-MEL-IAN cells were trypsinized and plated in 24 multi-well dishes at a density of 10 000 cells/well in melanoma medium. After three washes in PBS, the medium was changed to 1% charcoal-treated FCS (to remove the steroids present in FCS) and hormones at different concentrations dissolved in absolute ethanol (0.1% ethanol final concentration) were added. The media were changed every second day. At different times, cells were trypsinized and counted with an haemocytometer.

Statistical analysis

Significant differences from control values were evaluated by analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test.

Results

Scatchard plot analysis of androgen receptor and hormone specificity

In order to investigate the existence of ARs in these human melanoma cell lines, a whole-cell Scatchard analysis was performed with [^{3}H]R1881 ranging from 2.5 to 20 nM in melanoma medium (without serum) in the presence of TA (to avoid eventual binding of the tracer to PgRs). As can be seen in Figure 1, Scatchard plot analysis revealed a single binding component for both cell lines. For IIB-MEL-LES cells (Figure 1A), the apparent dissociation

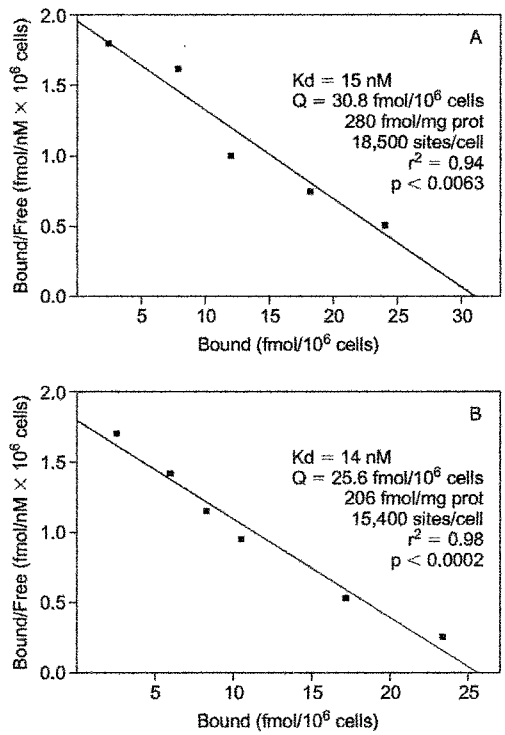

Figure 1. Scatchard plot analysis of ARs in IIB-MEL-LES (**A**) and IIB-MEL-IAN (**B**) whole cells at 37°C. Results shown are the mean ± SEM of triplicate determinations. This is a representative graph from three experiments.

constant (K_d) obtained was 15 nM, with a binding capacity of 30.8 fmol/10^6 cells, 280 fmol/mg total cell protein or 18 500 binding sites/cell. IIB-MEL-IAN cells (Figure 1B) showed a K_d of 14 nM, with a binding capacity of 25.6 fmol/10^6 cells, 206 fmol/mg total cell protein or 15 400 binding sites/cell.

The specificity of [^{3}H]R1881 binding was tested in order to assess if the lack of specificity previously found in IIB-MEL-J cells [6] was a common feature of human melanoma cell lines. As shown in Table 1, not only androgen (DHT and testosterone) and anti-androgen (hydroxyflutamide) but also non-androgenic compounds (progesterone, cortisol and DOCA) were able to compete significantly for [^{3}H]R1881 binding. E_2 showed a partial but significant reversion in IIB-MEL-LES and total reversion of binding in IIB-MEL-IAN.

Immunocytochemistry of steroid receptors in human melanoma cell lines, normal human tissue and human melanoma metastases

Immunocytochemistry of IIB-MEL-LES and IIB-MEL-IAN cells was performed with the monoclonal antibody anti-AR AN1-15 on cells grown as monolayers over glass slides or embedded in tissue inclusion medium (OCT). Figure 2 shows micrographs of steroid receptor staining in IIB-MEL-LES and IIB-MEL-IAN cells, demonstrating the presence of ARs and the absence of ERs in these cells. Similar negative results were obtained with PgRs (not shown).

As both the present and past studies have shown the presence of ARs in established cell lines, we decided to assay 10 biopsy specimens of melanoma metastases for the presence of this receptor. As shown in Table 2, all melanoma metastases analysed stained positively for ARs, the intensity of the staining being high in nine ot the 10 specimens.

Some normal tissues were also tested for ARs. As can be seen in Table 3, normal mammary gland was negative for ARs. When a Male patient's skin was analysed, the staining intensity was high in all types of keratinocytes and in the sweat glands, as well as in epithelial cells from typical androgen target organs such as the prostate gland and epididymis.

Effect of different hormones and anti-hormones on cell proliferation

Several hormones and anti-hormones were tested for their ability to stimulate cell proliferation in both

Table 1. Specificity of binding of [^{3}H]R1881 to whole cells. IIB-MEL-LES and IIB-MEL-IAN cells were incubated with 22 nM and 9 nM [^{3}H]R1881, respectively, as described in Materials and methods

Hormone	IIB-MEL-LES (d.p.m. ± SEM)			IIB-MEL-IAN (d.p.m. ± SEM)		
	100 ×	500 ×	1000 ×	100 ×	500 ×	1000 ×
Control	20 367 ± 2896	20 367 ± 2896	20 367 ± 2896	4978 ± 312	4978 ± 312	4978 ± 312
Testosterone	8400 ± 585***	7779 ± 430***		2957 ± 125***	2392 ± 96.3***	
DHT	8194 ± 996***	6374 ± 369***		3008 ± 406***	2488 ± 147***	
R1881	8383 ± 786***	8084 ± 654***		2405 ± 106***	1983 ± 133***	
Hydroxyflutamide			7597 ± 296***			2387 ± 236***
E_2		12 741 ± 1227**			3258 ± 200***	
Progesterone		8831 ± 230***			3059 ± 218***	
Cortisol		9569 ± 764***			3582 ± 123***	
DOCA		9197 ± 123***			3082 ± 68.5***	

**$P < 0.01$, ***$P < 0.001$ with respect to control by ANOVA followed by Tukey–Kramer multiple comparisons test.

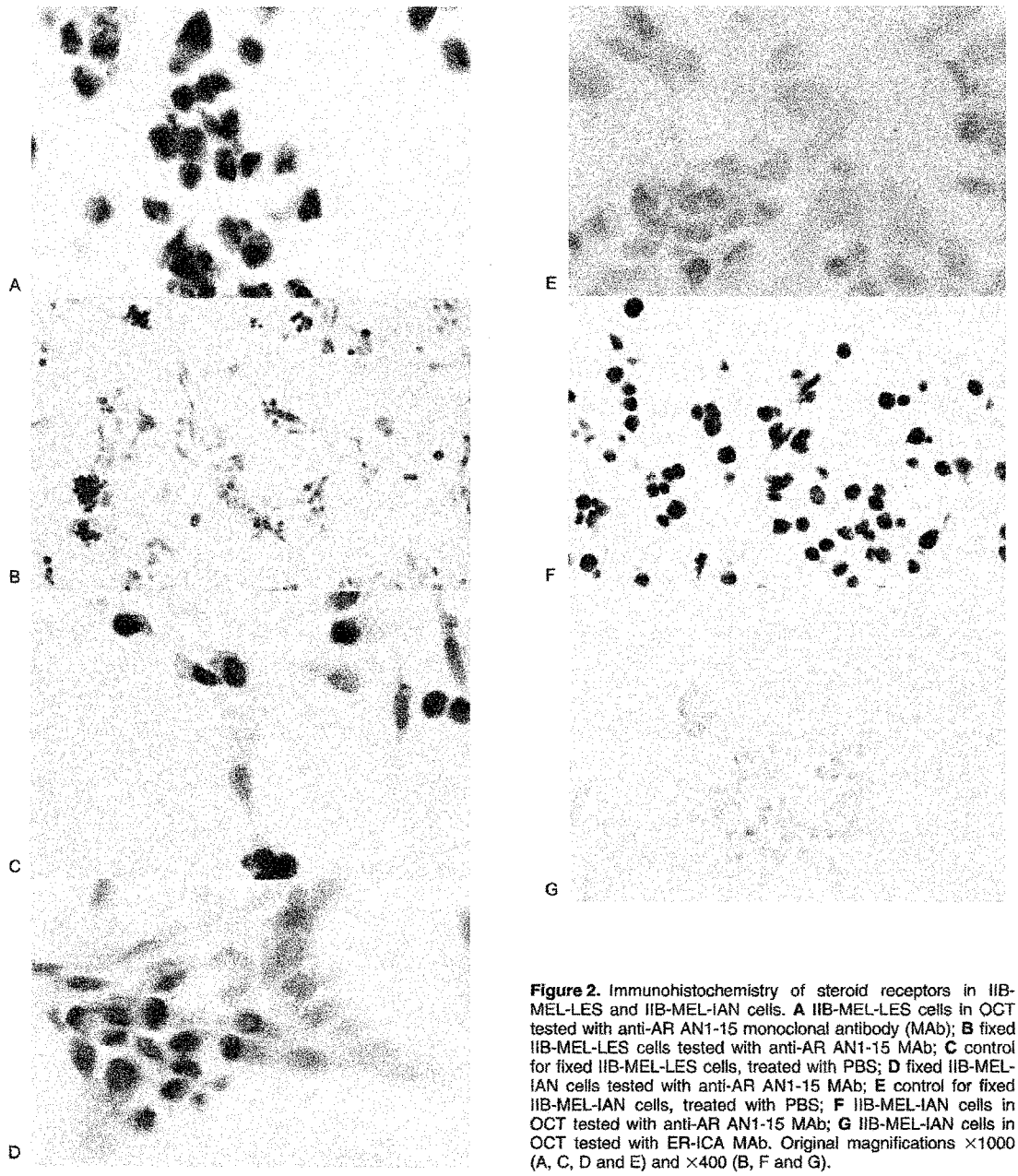

Figure 2. Immunohistochemistry of steroid receptors in IIB-MEL-LES and IIB-MEL-IAN cells. **A** IIB-MEL-LES cells in OCT tested with anti-AR AN1-15 monoclonal antibody (MAb); **B** fixed IIB-MEL-LES cells tested with anti-AR AN1-15 MAb; **C** control for fixed IIB-MEL-LES cells, treated with PBS; **D** fixed IIB-MEL-IAN cells tested with anti-AR AN1-15 MAb; **E** control for fixed IIB-MEL-IAN cells, treated with PBS; **F** IIB-MEL-IAN cells in OCT tested with anti-AR AN1-15 MAb; **G** IIB-MEL-IAN cells in OCT tested with ER-ICA MAb. Original magnifications ×1000 (A, C, D and E) and ×400 (B, F and G).

cell lines. The effect of different drugs on IIB-MEL-LES cell proliferation is shown on Figure 3. DHT was able to significantly stimulate cell proliferation at 10 nM. This increase was reversed by 4 μM hydroxyflutamide (Figure 3A), as expected, but also by 1 μM tamoxifen (Figure 3B). Growth enhancement was also found with E_2 (1 nM) (Figure 3C) and progesterone (1 nM) (Figure 3D). In both cases either hydroxyl-flutamide or tamoxifen reversed the effect. Figure 3E shows the enhancement produced by testosterone (20 nM) on these cells, this enhancement being reversed by either 4 μM hydroxyl-flutamide (Figure 3E) or 20 nM bicalutamide (Casodex) (Figure 3F). In summary, all the hormones have a

Table 2. Reactivity of anti-androgen receptor monoclonal antibody AN1-15 in melanoma specimens from patients

Patient	Sex	Cell positivity[a]	Intensity[b]
C.T.	Male	++++	High
G.B.	Male	++++	High
G.H.	Female	++++	High
M.B.	Male	++++	High
J.E.	Male	++++	High
J.L.	Male	+++	High
A.V.	Female	++++	High
O.O.	Male	++++	High
M.G.	Female	++	High
E.C.	Male	+++	Moderate

[a]Mean percentage of positive cells, where the total number of tumour cells is considered to be 100%, classified as + (0–25%), ++ (26–50%) +++ (51–75%) or ++++ (76–100%).
[b]Reaction intensity classified as low, moderate or high.

stimulatory effect, while the anti-hormones alone are similar to control values, independently of the kind of steroid involved, and they also reverse the stimulatory effects of the agonists.

In Figure 4 the results of treating IIB-MEL-IAN with the same hormones and anti-hormones as used IIB-MEL-LES are shown. The results are similar for both cell lines.

Discussion

The presence of ARs has been previously demonstrated by our group in the human melanoma cell line IIB-MEL-J, both by [^{3}H]R1881 binding and by immunocytochemistry [6]. However, the binding was atypical, with competition by non-androgenic compounds such as E_2, progesterone or cortisol. Moreover, androgen enhancement and anti-androgen reversion of cell proliferation could be replicated with E_2 and tamoxifen. It has been described that human solid tumours undergo multiple genetic changes as they progress from a near-normal state to aggressive malignancy [12]. Also, cell lines tend to change in culture. For instance, during *in vitro* cell culturing, prostate cancer LNCaP cells accumulate altered biological behaviours. These cells express the same level of functional ARs but exhibit a different responsiveness to androgen stimulation at later stages [13]. Moreover, a typical example of different tumoral cell lines expressing or not expressing receptors is the case of breast cancer cell lines. Whereas MCF-7, ZR-75-1 and T47-D are ER-positive, other cell lines such as MDA-MB-231 and HS 578T are ER-negative. Therefore, the expression of a receptor type in one cell line does not prove its expression in other cell lines, or in the original tumours. The study of the characteristics of ARs in other melanoma cell lines was consequently of interest. Similar results were found in the present study of two other human melanoma cell lines.

The antiproliferative activity of tamoxifen has also been described by others in three human melanoma cell lines via binding to type II ERs (nuclear-matrix associated ERs) [14]. We have not searched for type II ERs in these cells or samples because they were only tested by immunohistochemistry. In addition, ERβ were not analysed. However, ERα and ERβ cannot be distinguished in binding assays. We did not measure the binding of [^{3}H]E_2 in these two cell lines. Nevertheless, we have already published [6] that in IIB-MEL-J cells, in the absence of radio-inert DHT, specific binding of [^{3}H]E_2 was found. However, in the presence of radio-inert DHT, specific binding was suppressed, suggesting that this binding may be due to interaction with an AR. Therefore, and considering that the cell proliferation experiments

Table 3. Reactivity of anti-androgen receptor monoclonal antibody AN1-15 in normal tissues

Tissue	Sex	Cell type	Cell positivity[a]	Intensity[b]
Mammary gland	Female	Epithelium	—	—
		Fibroblasts	—	
Skin	Male	Superficial keratinocytes	++++	High
		Intermediate keratinocytes	+++/++++	High
		Basal keratinocytes	+++/++++	High
		Dermis	—	—
		Sweat glands	++	High
Prostate gland	Male	Epithelium	++/++++	High and moderate
		Fibroblasts	—	
Epididymis	Male	Epithelium	++/++++	Low and moderate
		Fibroblasts	—	
		Smooth muscle	—	

[a]Mean percentage of positive cells, where the total number of tumour cells is considered to be 100%, classified as + (0–25%), ++ (26–50%) +++ (51–75%) or ++++ (76–100%).
[b]Reaction intensity classified as low, moderate or high.

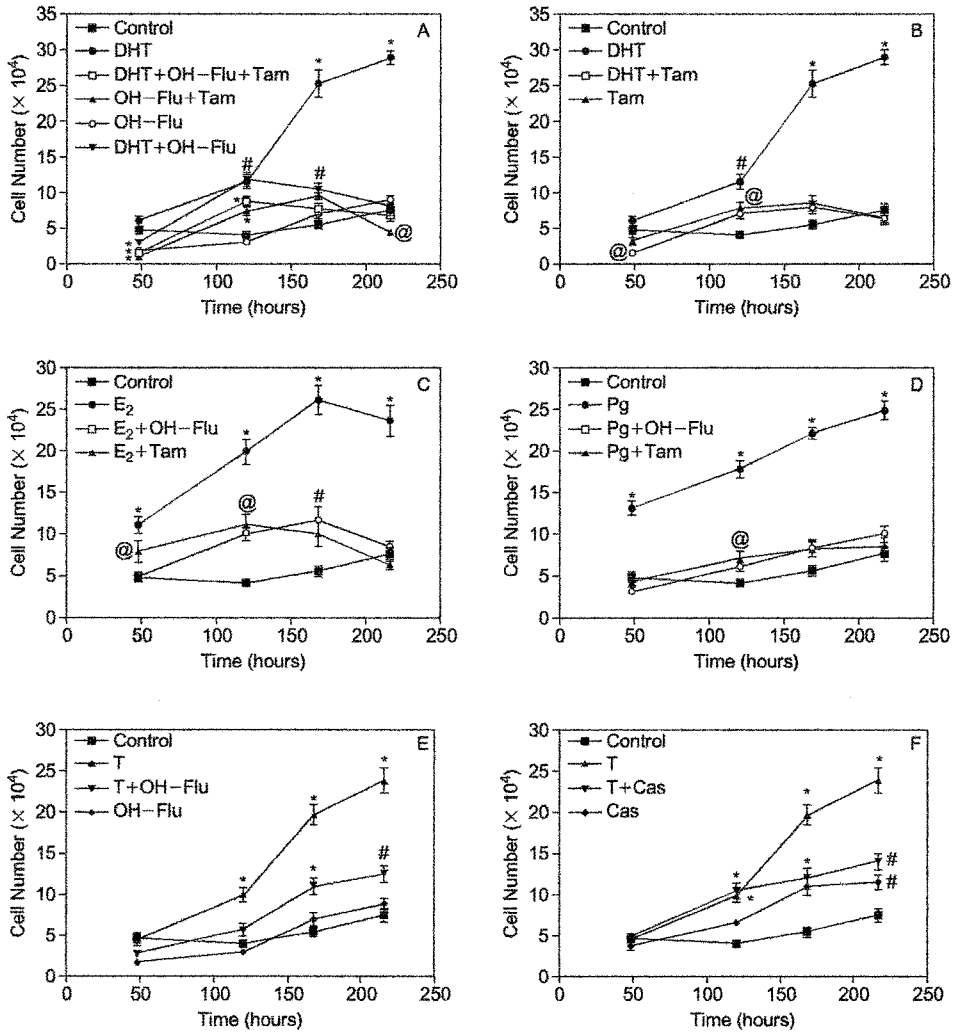

Figure 3. Effect of hormones and anti-hormones on proliferation of IIB-MEL-LES cells. **A** Effect of medium (control), 10 nM DHT, and 4 μM hydroxyflutamide (OH-Flu) alone or in combination with DHT or 1 μM tamoxifen (Tam) or both DHT and tamoxifen. **B** Effect of medium (control), DHT, and tamoxifen (Tam) alone or in combination with DHT. **C** Effect of medium (control) and 1 nM E_2 alone or in combination with hydroxyflutamide (OH-Flu) or tamoxifen (Tam) at the concentrations stated before. **D** Effect of medium (control) and 1 nM progesterone (Pg) alone or in combination with hydroxyflutamide (OH-Flu) or tamoxifen (Tam). **E** Effect of medium (control), 20 nM testosterone (T) and hydroxyflutamide (OH-Flu) alone or in combination with testosterone. **F** Effect of medium (control), testosterone, and 20 nM bicalutamide (Casodex; Cas) alone or in combination with testosterone. Each point represents the mean ± SEM of three determinations. $^{*}P < 0.001$, $\#P < 0.01$ and $^{@}P < 0.05$ with respect to the control as evaluated by ANOVA followed by Tukey's test. This figure is representative of two experiments.

were consistent with the binding assays, we believe that the effect of oestrogens was due to interaction with an AR.

The peculiar behaviour of the AR could be due to a mutation of the steroid-binding domain, as described for LNCaP prostate cancer cells [15]. However, in the mutation described in these cells, oestrogen, progesterone and anti-androgens all behave as agonists, whereas in the three melanoma cell lines, the hormones stimulate cell proliferation while the anti-hormones reverse this effect. The possibility of three independent mutations in these cell lines does not seem very likely, although in advanced prostate cancer the same codon mutation of the AR described in LNCaP cells was found in six out of 24 specimens [16]. A different hypothesis other than

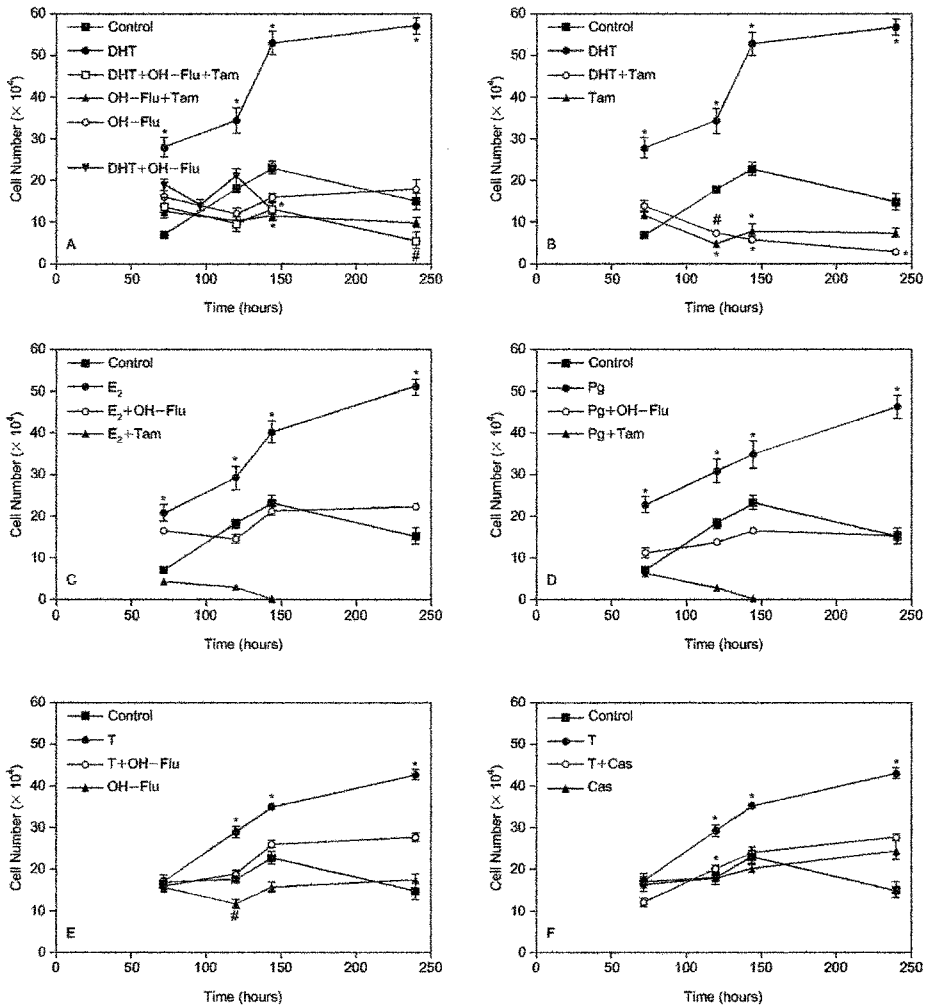

Figure 4. Effect of hormones and anti-hormones on proliferation of IIB-MEL-IAN cells. **A** Effect of medium (control), 10 nM DHT, and 4 μM hydroxyflutamide (OH-Flu) alone or in combination with DHT or 1 μM tamoxifen (Tam) or both DHT and tamoxifen. **B** Effect of medium (control), DHT, and tamoxifen (Tam) alone or in combination with DHT. **C** Effect of medium (control) and 1 nM E_2 alone or in combination with hydroxyflutamide (OH-Flu) or tamoxifen (Tam) at the concentrations stated before. **D** Effect of medium (control) and 1 nM progesterone (Pg) alone or in combination with hydroxyflutamide (OH-Flu) or tamoxifen (Tam). **E** Effect of medium (control), 20 nM testosterone (T) and hydroxyflutamide (OH-Flu) alone or in combination with testosterone. **F** Effect of medium (control), testosterone, and 20 nM bicalutamide (Casodex; Cas) alone or in combination with testosterone. Each point represents the mean ± SEM of three determinations. $^*P < 0.001$ and $\#P < 0.01$ with respect to the control as evaluated by ANOVA followed by Tukey's test. This figure is representative of two experiments.

the 'hot spot' for the appearance of a similar behaviour in different cell lines could be the influence of growth factors or second messengers on ARs. It has been described that growth factors such as epidermal growth factor or insulin growth factor-I can enhance the ligand-dependent transactivation property of the AR, and even activate it in a ligand-independent manner (reviewed by Cato and Peterziel [17]). With respect to second messengers, it has been reported [18] that cAMP response element binding protein (CREB) binding protein is able to enhance AR transcription and that this coactivator serves as an integrator between androgen-mediated and other signalling pathways. It may also be speculated that the lack of specificity of ARs found in melanoma cells could be due to non-genomic actions of hormones, such as an increase in intracellular calcium. Alternatively, modulators of protein

kinase A could be activating ARs alone or potentiating the ligand-dependent transactivating function of ARs, as recently reviewed [17].

A novel signalling pathway has been described for steroids in the prostate involving sex hormone binding globulin [19]. In dog prostate cells in culture, steroids such as E_2, in the presence but not in the absence of SHBG, were able to stimulate an androgen-dependent protein, and this effect was antagonized by an anti-androgen or a competitive inhibitor of binding to SHBG. This action was also found with forskolin or 8-Br-cAMP, and the authors suggest that ARs can be activated, even in the absence of androgens, by natural steroids that activate the cAMP pathway via AR phosphorylation. It is plausible that activation of the SHBG-R_{SHBG} pathway could lead to androgen-independent AR activation via stimulation of protein kinase A. This mechanism could eventually explain the similarity between the stimulation of cell proliferation by testosterone, DHT and non-androgenic hormones. However, the possibility of this pathway of cell proliferation is not likely, since binding assays with [^{3}H]R1881 showed similar specificity, and it has been described [20] that this ligand does not bind to SHBG or other steroid-binding proteins.

[^{3}H]R1881 binding was tested in whole cells, but the localization of receptors in the nucleus, cytoplasm or cell membrane cannot be established with this technique. However, when the cells were tested by immunocytochemistry, the receptors were localized in the nucleus and were recognized by a usual AR antibody. For these reasons, a nuclear AR may be present in these cells, even if the specificity is not typical. The lack of specificity in both cell proliferation and androgen binding suggest the presence of an atypical AR.

As shown by Scatchard analysis, a single binding site was found for both IIB-MEL-LES and IIB-MEL-IAN human melanoma cells. It has been described [21] that the AR is expressed in a variety of tissues as two different forms transcribed from a single gene. However, the A form is present at relatively low and constant levels. The two isoforms are detected by their different molecular weights. In the present study the presence of this receptor was detected by binding assay. As the androgen-binding domain is the same in both isoforms [22], the binding activity is likely to be similar and the K_d should be alike. Thus the detection of a single binding component by Scatchard analysis is not unexpected. The antibody used also recognizes the androgen-binding domain [10] and should stain both isoforms.

The presence of ARs was confirmed in both cell lines by immunocytochemistry, and significant staining was seen for both lines. Moreover, the presence of ARs was also demonstrated by immunocytochemistry in all the biopsy specimens from melanoma metastases. This is a point to highlight, because the importance of the presence of ARs in all three human melanoma cell lines (IIB-MEL-J, IIB-MEL-LES and IIB-MEL-IAN) is reinforced by AR positivity in all the specimens tested. Recently, studies of ARs in naevus sebaceus of Jadassohn have been reported [23]. AR positivity was seen in the sebaceous glands, in eccrine glands with and without apocrine change, and rarely in keratinocytes in the sebaceous naevi. There were no significant differences in the staining location or pattern between the age groups or sexes. Normal skin showed similar staining in the sebaceous glands but did not show staining of the eccrine glands or keratinocytes. It has also been described [24] that a significant proportion of plasma-borne testosterone is converted in androgen target tissues, such as the skin, to the more potent androgen DHT by the steroid 5α-reductase type 1 and type 2 isoenzymes. DHT binds to the nuclear AR with much greater affinity than testosterone. Therefore the simultaneous presence of the receptor and the converting enzyme would enhance the importance of demonstration the presence of ARs. We have not investigated the presence of 5α-reductase in these human melanoma cell lines.

This study shows clear stimulation of cell proliferation in both cell lines by androgens as well as other steroids. Moreover, the anti-hormones tested reversed the enhancement of cell proliferation by any hormone. Similarly, our group has previously described [6] that tamoxifen was able to antagonize the stimulatory effect of DHT on cell proliferation in IIB-MEL-J cells, and steroids were able to compete non-specifically with [^{3}H]R1881 binding. Likewise, both E_2 and progesterone are able to stimulate cell proliferation in IIB-MEL-J cells (data not shown). This effect, together with the lack of specificity of [^{3}H]R1881 binding, suggests that the AR in melanoma cells is atypical.

Acknowledgements

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Fundación Alberto J. Roemmers, the Fundación Sales, and the Fundación para la Investigación y Prevención del Cáncer (FUCA), Argentina. M.I. Capurro is a Fellow of the Fundación Sales; I.A. Lüthy, R.S. Calandra and J. Mordoh are Research

Career Members from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

References

1. MacKie RM. Incidence, risk factors and prevention of melanoma. *Eur J Cancer* 1998; **34**(Suppl 3): S3–S6.
2. Chang AE, Karnell LH, Menck HR. The National Cancer Data Base report on cutaneous and noncutaneous melanoma. *Cancer* 1998; **83**: 1664–1678.
3. Fisher RI, Neifeld JP, Lippman ME. Estrogen receptors in human malignant melanoma. *Lancet* 1976; **ii**: 337–338.
4. Rusthoven JJ. The evidence for tamoxifen and chemotherapy as treatment for metastatic melanoma. *Eur J Cancer* 1998; **34**(Suppl 3): S31–S36.
5. Rampen FHJ, Mulder JH. Malignant melanoma: an androgen-dependent tumour? *Lancet* 1980; **i**: 562–565.
6. Morvillo V, Luthy IA, Bravo AI, Capurro MI, Donaldson M, Quintans C, *et al.* Atypical androgen receptor in the human melanoma cell line IIB-MEL-J. *Pigment Cell Res* 1995; **8**: 135–141.
7. Kairiyama C, Slavutsky I, Larripa I, Morvillo V, Bravo AI, Bover L, *et al.* Biological, immunocytochemical, and cytogenetic characterization of two new human melanoma cell lines: IIB-MEL-LES and IIB-MEL-IAN. *Pigment Cell Res* 1995; **8**: 121–131.
8. Luthy IA, Begin D, Labrie F. Mediation by the androgen receptor of the stimulatory and antiandrogenic actions of 17β-estradiol on the growth of androgen-sensitive Shionogi mammary carcinoma cells in culture. *Endocrinology* 1988; **123**: 1418–1424.
9. Asslin J, Melançon R, Gourdeau Y, Labrie F, Bonne C, Raynaud JP. Specific binding of [^{3}H]-methyltrienolone to both progestin and androgen binding components in human benign prostatic hypertrophy (BPH). *J Steroid Biochem* 1979; **10**: 483–486.
10. Chang C, Wheeelan C, Popovich T, Kokontis J, Liao S. Fusion proteins containing androgen receptor sequences and their use in production of poly- and monoclonal anti-androgen receptor antibodies. *Endocrinology* 1989; **125**: 1097–1099.
11. Ballare C, Bravo AI, Sorin I, Guman N, Schiaffi JA, Yomha R, *et al.* The expression of progesterone receptors coincides with an arrest of DNA synthesis in human breast cancer. *Cancer* 1991; **67**: 1352–1358.
12. Smith CA, Pollice AA, Gu L-P, Brown KA, Singh SG, Janocko LE, *et al.* Correlations among p53, Her-2/neu, and ras overexpression and aneuploidy by multiparameter flow cytometry in human breast cancer: evidence for a common phenotypic evolutionary pattern in infiltrating ductal carcinomas. *Clin Cancer Res* 2000; **6**: 112–126.
13. Lin MF, Meng TC, Rao PS, Chang C, Schonthal AH, Lin FF. Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines. *J Biol Chem* 1998; **273**: 5939–5947.
14. Piantelli M, Maggiano N, Ricci R, Larocca LM, Capelli A, Scambia G, *et al.* Tamoxifen and quercetin interact with type II estrogen binding sites and inhibit the growth of human melanoma cells. *J Invest Dermatol* 1995; **105**: 248–253.
15. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, *et al.* A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* 1990; **173**: 534–540.
16. Gaddipati JP, McLeod DG, Heidenberg HB, Sesterhenn IA, Finger MJ, Moul JW, *et al.* Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancer. *Cancer Res* 1994; **54**: 2861–2864.
17. Cato ACB, Peterziel H. The androgen receptor as mediator of gene expression and signal transduction pathways. *Trends Endocrinol Metab* 1998; **9**: 150–154.
18. Aarnisalo P, Palvimo JJ, Jänne OA. CREB-binding protein in androgen receptor-mediated signalling. *Proc Natl Acad Sci USA* 1998; **95**: 2122–2127.
19. Ding VDH, Moller DE, Feeney WP, Didolkar V, Nakhla AM, Rhodes L, *et al.* Sex hormone-binding globulin mediates prostate androgen receptor action via a novel signaling pathway. *Endocrinology* 1998; **139**: 213–218.
20. Bonne C, Raynaud J-P. Methyltrienolone, a specific ligand for cellular androgen receptors. *Steroids* 1975; **26**: 227–232.
21. Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are expressed in a variety of human tissues. *Mol Cell Endocrinol* 1996; **120**: 51–57.
22. Wilson CM, McPhaul MJ. A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proc Natl Acad Sci USA* 1994; **91**: 1234–1238.
23. Hamilton KS, Johnson S, Smoller BR. The role of androgen receptors in the clinical course of nevus sebaceus of Jadassohn. *Mod Pathol* 2001; **14**: 539–542.
24. Andersson S. Steroidogenic enzymes in skin. *Eur J Dermatol* 2001; **11**: 293–295.

(Received 5 November 2001; accepted in revised form 13 May 2002)