Abstract. Background/Aim: Growing evidence links stress hormones with development and progression of various cancer types. The aim of this study was to assess susceptibility of cutaneous and uveal melanoma cells to adrenaline (AD). Materials and Methods: The expression of β-2-adrenergic receptor in primary cutaneous (FM-55-P), primary uveal (92-1, Mel202) and metastatic cutaneous (A375) melanoma cells was estimated at mRNA, protein and cell surface levels. The impact of AD on cell proliferation and migration was also studied. Results: The expression of β-2-adrenergic receptor was cell line-dependent. Adrenaline treatment caused a slight stimulation of melanoma cell proliferation and activation of matrix metalloproteinases. Adrenaline-treated uveal melanoma cells showed an increased migration rate, whereas, in cutaneous melanoma cells, no changes or even lower migration speed were observed. Conclusion: Melanoma cell susceptibility to AD varies depending on origin and progression stage. Metastatic cutaneous melanoma cells were found to be less responsive to AD than primary cutaneous and uveal melanoma cells.

The high mortality rate associated with melanoma is attributed to the high invasiveness of tumour cells that becomes apparent very early during disease progression (1). Among the classical risk factors, such as ultraviolet (UV) exposure, sunburn, fair skin type, gender or age (2), psychological stress is also considered to be an important factor in melanoma development (3, 4). There is growing evidence showing that the chronic stress and depression experienced by oncological patients accelerate the course of disease progression (5). Stress-related immunosuppression is suggested to be the main cause of an increased tumour growth and faster spread of metastatic cancer cells (6), including melanoma (7). Nevertheless, it has been recently accepted that stress effectors, such as chemokines and neurotransmitters (adrenaline (AD)) can also directly affect cancer cells and, thereby, influence disease/tumour progression (8, 9). Cancer cell dissemination is a multistep process (10) during which the cells are not only under the influence of local microenvironment, but also tightly regulated by intracellular signalling pathways. Many cancer cells have been shown to express adrenergic receptors and to be sensitive to catecholamine stimulation (11). The involvement of β-adrenergic receptor (ADRB) signaling in cancer progression has been confirmed with the use of β-blockers. The antiproliferative and pro-apoptotic effects of propranolol, a non-selective β-blocker for ADR1B and ADR2B, have been observed in hemangioendotheliomas and angiosarcomas (12), gastric cancer (13), breast cancer (14) and neuroblastoma (15). In a melanoma mouse model, the biphasic effect of propranolol on tumour growth has been shown, suggesting that tumour growth is inhibited by vasoconstrictive doses of propranolol, while vasodilating doses do not exert such effect (16). Moreover, propranolol has been shown to significantly reduce melanoma cell migration (17). In a breast cancer mouse model, propranolol lowers cell migratory potential, thereby blocking distant metastasis formation (18). Furthermore, until now, only a few studies have examined the molecular effects of AD on melanoma cells (17, 19).

In the present study, we examined the effect of AD stimulation on four melanoma cell lines that differ in their origin: cutaneous (CM), i.e. A375 and FM-55-P (metastatic and primary, respectively), as well as uveal (UM), i.e. 92-1 and Mel202 (both primary). We examined the influence of AD stimulation on ADR2B receptor expression on melanoma cell surface, as well as its impact on cell proliferation and migration. The ADR2B, which is the potent
AD receptor (20), is known to be widely expressed in tissues and its extensive expression has been reported in cancer cells (21), including melanoma (17). We also investigated the activity of matrix metalloproteinases (MMPs) MMP-2 and MMP-9 reflecting the invasive ability of tumour cells.

Materials and Methods

Chemicals. AD and gelatin type B from bovine skin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-beta-2-adrenergic receptor (ADR2B) antibody was obtained from Abcam (Cambridge, UK). SplitKits were purchased from Seralab (West Sussex, UK). Goat anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Western Bright Quantum HRP substrate was a product of Advansta (Menlo Park, CA, USA). High Pure RNA Isolation Kit and Transcriptor High Fidelity cDNA Synthesis Kit were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Foetal bovine serum, RPMI-1640 Glutamax-I®, Alamar Blue, SYBR® Green Master Mix, normal rabbit IgG isotype control and secondary donkey anti-rabbit antibody conjugated with AlexaFluor488 were purchased from Life Technologies (Carlsbad, CA, USA). Specific primers for Real-Time polymerase chain reaction (PCR) were obtained from Oligo.pl (Warsaw, Poland). All remaining chemicals were of analytical grade, commercially available.

Cell culture and treatment. A375 cell line (malignant CM) was purchased from ATCC (Manassas, VA, USA). Primary CM FM-55-P cell line, primary UM 92-1 cells and Mel202 cell line were obtained from ESTDAB Melanoma Cell Bank (Tübingen, Germany). The cells were maintained in RPMI-1640 Glutamax-I™ medium (GibcoBRL, Paisley, UK) supplemented with 10% (v/v) heat-inactivated foetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml) solution. The cells were grown in monolayers at 37°C in a humidified atmosphere containing 5% CO2. All experiments were initiated in sub-confluent cultures. In some experiments, cells were treated with AD (0.1, 1, 10, 100 μM) for 12-24 h prior to assays.

Cell viability assay. Confluent cells were detached, pelleted and re-suspended in assay medium. Subsequently, cells were counted and seeded into 96-well plates at the density of 1x104 cells/100 μl. On the following day, medium was changed and cells were incubated with different doses of AD. After 12 h of incubation, 10% Alamar Blue was added to each well. The mixture was incubated for 3 h and, then, fluorescence intensity was measured at 560/595 nm.

Real-Time PCR. Total RNA was extracted from cell lysates and 1 μg of total RNA was reverse-transcribed. The Real-Time PCR reactions were performed for 40 cycles of denaturation (15 sec, 95°C), annealing/elongation (1 min, 60°C). Amplification of GAPDH mRNA was used as an internal control of efficiency of the reaction. The oligonucleotide sequences for ADR1B, ADR2B and GAPDH were as follows: for ADR1B, 5'CCTCGTCGCGATGCTCTTC' and 5'GCAAGCCTGCTGATTTCACA'3'; for ADR2B, 5'AGAGCCTCAAGATCATCAGCA'3' and 5'TAGCAGTTGATGGCTCTTC' for GAPDH, 5'CAGCCTCAAGATCATCAGCA'3' and 5'GTCTTCTGGTGTCAGATGAT'3'. The reaction results were analysed with a use of StepOne Software v 2.0 software (Thermo Fisher Scientific, Waltham, MA, USA).

Flow cytometry. The expression of ADR2B in control and AD-stimulated cells was assessed by flow cytometry. Briefly, the cells were grown to sub-confluence and, then, either treated or not for 24 h with different doses of AD. Subsequently, cells were harvested with the use of SplitKits and diluted to 1.5x106/ml of PBS. Then, the cells were stained with either 1 μl of rabbit anti-human antibody against ADR2B (1 mg/ml) or 0.25 μl of normal rabbit IgG (negative control) (3 mg/ml) for 45 min at 4°C. After washing, cells were stained with 0.5 μl of donkey anti-rabbit Alexa488-conjugated antibody (2 μg/ml) for 45 min at 4°C and washed again. The fluorescence of stained cells was measured by a FACScalibur flow cytometer (BD Biosciences, San Diego, CA, USA). Three independent experiments were carried out.

Wound-healing assay. Wound-healing assay was performed in 6-well culture plates that were either covered or not with fibronectin (FN). Briefly, melanoma cells were grown to confluence and, then, the cell-coated surface was scraped with a pipette tip in a single stripe. Subsequently, the surface was washed twice with PBS, covered with complete medium and wounds were allowed to heal for 24 h at 37°C in a humidified atmosphere. In some experiments, wound healing was performed in complete culture medium containing AD. Migration of cells into wounded areas was evaluated with an inverted microscope and photographed in 10 separated fields using Zeiss AxioVision Rel.4.8 image analysis software (Carl Zeiss Microscopy GmbH, Jena, Germany). The average extent of wound closure was evaluated by multiple measurements of the wound immediately after scraping and after 24 h. Three independent experiments were carried out and all data are given as percentage of migration compared to untreated (control) cells migrating on plastic surface.

Zymography. The serum-free culture media, conditioned by CM and UM cells either treated or not with AD, were normalized for protein concentration, mixed with Laemmli sample buffer without reducing agents (10 μg of total protein per line) without boiling. Then, samples were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels containing copolymerised gelatin (SDS)-polyacrylamide gels containing copolymerised gelatin at final concentration of 1%. After separation, the gels were washed in a solution of 2.5% Triton X-100 (2×15 min) to remove SDS and after that in water (2×5 min). In the next step, the gels were incubated for 24 h at 37°C in 50 mM Tris/HCl, pH 8, containing 0.15 M NaCl, 5 mM CaCl2 and 0.02% sodium azide. Then, the gels were stained in 0.1% Coomassie Brilliant Blue R250 in a solution of 50% methanol and 10% acetic acid and, finally, destained. The dried gels were photographed with the use of a ScionImage software (Scion Corp., Frederick, MD, USA). The semiquantitative analysis of obtained bands intensity was conducted with the use of UVImap V.99 software (UVTec, Cambridge, UK).

Cell lyase preparation. Cell pellets were homogenized in extraction buffer (50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and protease inhibitor cocktail) by sonification (UP50H; Hielser Ultrasonics GmbH, Teltow, Germany), followed by extraction for 30 min on ice in the same buffer containing additionally 0.03% protease inhibitor cocktail and 1% Triton X-100. Finally, cell extracts were clarified by centrifugation at 18,000 × g for 25 min. Protein concentration was determined in the supernatants according to Peterson (22).
between the control and the AD-treated cells in all analysed cell lines. However, a significant difference in the percentage of cells, only a small percentage of A375 cells was ADR2B-positive. Contrary to the observation related to RFI (17), the reported RFI value was low, suggesting presence of a small amount of the ADR2B receptor on the cell surface. These observations corresponded to the results of mRNA expression analysis. Moreover, referring to the cell surface expression of ADR2B receptor differed among the melanoma cell lines. The highest RFI value was detected in Mel202 cells and the lowest in FM-55-P cells. Also, in the case of A375 cells, the reported RFI value was low, suggesting presence of a small amount of the ADR2B receptor on the cell surface. These observations corresponded to the results of mRNA expression analysis. Moreover, referring to the percentage of cells, only a small percentage of A375 cells was ADRB2-positive. Contrary to the observation related to RFI value, Mel202 cell line had the smallest population of ADR2B-positive cells, whereas the FM-55-P cell line had the highest value (17). These results were consistent with previously published observations (17); therefore, further analysis refers entirely to ADR2B receptor expression and action. We found that 92-1 cells showed the highest level of ADR2B mRNA expression among all analysed melanoma cell lines. Western Blot analysis confirmed the expression of ADR2B at the protein level in all analysed cell lines (Figure 1).

The flow cytometry results demonstrated that the density of the cell surface expression of ADR2B receptor differed among the melanoma cell lines. The highest density, expressed by relative fluorescence intensity (RFI) value, was detected in Mel202 cells and the lowest in FM-55-P cells. Also, in the case of A375 cells, the reported RFI value was low, suggesting presence of a small amount of the ADR2B receptor on the cell surface. These observations corresponded to the results of mRNA expression analysis. Moreover, referring to the percentage of cells, only a small percentage of A375 cells was ADRB2-positive. Contrary to the observation related to RFI value, Mel202 cell line had the smallest population of ADR2B-positive cells, whereas the FM-55-P cell line had the highest (Figure 2A and B). To gain a further insight into the expression of ADR2B in melanoma cells, the influence of adrenergic stimulation on its cell surface expression was determined. There were no statistically significant differences in RFI values between the control and the AD-treated cells in all analysed cell lines. However, a significant difference in the percentage of ADR2B-positive cells after AD stimulation was observed in primary FM-55-P and 92-1 cells.

Results

ADR2B expression in melanoma cells after AD stimulation. First, we assessed the expression of ADR1B (ADRB1) and ADR2B (ADRB2) genes in quantitative assay, using GAPDH mRNA as an internal control. It was shown that the ADR1B mRNA expression level (data not shown) was negligible in comparison to the values for ADR2B mRNA (Figure 1). These results were consistent with previously published observations (17); therefore, further analysis refers entirely to ADR2B receptor expression and action. We found that 92-1 cells showed the highest level of ADR2B mRNA expression among all analysed melanoma cell lines. Western Blot analysis confirmed the expression of ADR2B at the protein level in all analysed cell lines (Figure 1).

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Statistical analysis. Results are expressed as mean±standard deviation. Statistical analysis was performed with the use of Duncan’s new multiple range test or Student’s t-test and p-values of less than 0.05 were considered statistically significant. All data are the results of at least three separate experiments.

The effect of AD treatment on matrix metalloproteinase activity. In view of Moretti et al.’s (17) observations concerning the altered expression of active forms of MMP-2 and MMP-9 in melanoma cell lines cultured in the presence of catecholamines, gel zymography to examine matrix metalloproteinases was conducted (Figure 3). In all analysed cells, MMP-2 activity was detected. Moreover, the activity of pro-MMP-9 was reported only in primary melanoma cell lines and the intensity of the corresponding band was the most pronounced in 92-1 cells. In A375 cells, this form was not detected. A diverse MMP-2 activation after AD treatment was observed in A375, 92-1 and Mel202 cell lines but not in FM-55-P cells. The highest AD dose (100 μM) caused significant MMP-2 activation relative to control. The other applied doses had no effect or slightly decreased the level of the active MMP-2 form in A375 cells. In Mel202 cells, AD stimulation mostly increased the level of activated MMP-2. In 92-1 cells, there was an opposite effect: significantly decreased levels of the active form of MMP-2 were detected in case of AD treatment, regardless of the dosage used. FM-55-P cells showed no statistically significant differences in MMP-2 activation between control and AD-stimulated cells (Figure 3). In AD-stimulated primary melanoma cells, all doses administered caused a weak MMP-9 expression, while, in A375 cells, only 100 μM of AD resulted in induction of this MMP form. Additionally,
a band of 130 kDa was visualized only in both CM cell lines. This band may be attributable to the MMP-9/tissue inhibitor of metalloproteinases-1 (TIMP-1) complex, previously described (24); however, this hypothesis needs further verification. Its marked presence in samples originated from A375 cells may provide an explanation of the weak intensity of the bands representing pro-MMP-9 protein in this cell line.

**AD stimulation affects the proliferation and migration properties of CM and UM cells in various ways.** The evidence relating the role of stress in cancer progression is contradictory. Both increased and decreased rates of cancer cell proliferation, migration, invasion and tumour growth after catecholamine stimulation have been reported (25). Such observation has been made for various types of cancer, including breast (26), pancreatic (27) and others. To better understand the functional role of AD stimulation in melanoma cells, we measured proliferation (Figure 4) and migration rates (Figure 5). It was shown that primary melanoma cells were more susceptible to AD treatment; they responded mostly by lowering their proliferation rate. In the case of Mel202 cells, their proliferation rate was lower after application of 1 or 10 μM of AD, whereas 92-1 cells showed a slight decrease in proliferation after treatment with 0.1, 1 and 10 μM AD. The most pronounced effect was observed in FM-55-P cell line: all applied AD doses resulted in decreased cell proliferation. On the contrary, A375 cells responded by increasing their proliferation rate only, however, at the highest applied AD dose (100 μM) (Figure 4).

Subsequently, we investigated the effect of AD on melanoma cell migration as estimated in assays of wound healing on a plastic or FN-coated surface (Figure 5A-C). Previously reported wound-healing experiments in the herein analysed melanoma cell lines have revealed that the extent of wound closure on FN was over 70% for A375 (28), about 18% for FM-55-P cells and about 36% for both UM cell lines (29). First, we determined the role of AD in A375 cells migration rate on FN. Our results revealed that A375 cells migrated slower on FN after administration of all AD doses employed, while, on plastic, there was a difference observed only in the case of 10 μM of AD (Figure 5A). Then, based on our observation that 10 μM of AD was the only dose that decreased the proliferation to comparable level in all analysed primary melanoma cell lines, we tested the migration rate of melanoma cells on FN at this specific concentration (Figure 5B). Obtained results showed that FM-55-P cells migrated faster when seeded on the FN-coated surface, as it was expected, independently of AD treatment. The response of UM cells was consistent as both 92-1 and Mel202 cells markedly increased their migration speed after AD treatment.

**Discussion**

During the last several years, efforts to include psychological stress among risk factors generally accepted to be conducive to melanoma progression have been observed. Clinical findings that β-blockers might inhibit cancer progression (12, 14, 15, 30, 31) have sparked hopes for more effective cancer treatment. These studies have also inspired numerous in vivo and in vitro studies focused on elucidation of the mechanisms of catecholamines’ action in cancer. As various cancers, including melanoma, are concerned, a positive correlation between the level of stress in cancer patients and progression of disease has been demonstrated (8, 32). However, attempts to explain the molecular mechanisms of AD action in cancer progression (33-35) have still not brought clear conclusion(s) despite thorough investigation.
The adrenergic stimulation is suggested to accelerate cancer progression via stimulation of angiogenesis (36), regulation of MMP expression and activation (37), as well as by increased tumour cell proliferation (26). In melanoma tissues and cell lines, the expression of ADR2B has been previously reported (17, 38, 39). In the study of Yang et al. (38), the expression of ADR2B has been confirmed in the majority of analyzed melanoma tissue biopsies. Additionally, analysis of melanoma tissue samples performed by Moretti et al. (17) has shown higher ADR2B expression level in malignant biopsies. Further insight into ADR2B expression in melanoma biopsies have suggested its prognostic value in course of melanoma and demonstrated positive correlation between elevated ADR2B expression in patients’ tissue samples and poor survival rate (39). Additionally, it has been shown that metastatic melanoma A375 cells from primary tumour exhibited higher expression of ADR2B, both at mRNA and protein level than malignant melanoma Hs-29-4T cells from secondary tumour (17). Our present results revealed that melanoma cells, despite their origin and malignancy, exhibited both ADR2B mRNA expression and corresponding protein. Our observations were consistent with a previous study by Moretti et al. (17) showing that ADR2B expression in melanoma cells decreases in relation to growing metastatic potential of tumour cells.

![Image](image-url)

Figure 3. Abundant activation of metalloproteinase (MMP)-2 in melanoma cell lines. MMPs’ activation was determined by zymography in CM and UM cells treated with adrenaline in selected doses. Semi-quantitative analysis of MMP-2 band intensity was performed with the use of UVImap software. Values are expressed as means±standard deviation of three independent experiments. Statistically significant differences are asterisked (Duncan’s test, p<0.05).
Moreover, our results are consistent with the observation concerning ADR2B expression in breast cancer cells. Gargiulo et al. (26) have shown that the non-tumourigenic MCF-10A cells possess generally higher level of ADRB than tumour MCF-7 cells. Regarding their previously published results, they have suggested that this observation refers mainly to ADR2B. These authors have also reported that, in non-tumourigenic cells, AD treatment resulted in decreased proliferation and migration rate and higher cellular adhesion, whereas, in tumour cells, the effect was opposite, i.e. AD stimulation accelerated proliferation and migration of cancer cells (26). Our results showed, for the first time, that primary melanoma cell proliferation rate is lower after AD stimulation. Additionally, AD treatment caused an increase in proliferation of metastatic melanoma A375 cells; however, this effect was observed only in the case of the highest AD dose. Our observations were in line with the results previously reported by Yang et al. (38) who have shown noradrenaline-dependent (acting via ADRB) increase in proliferation rate of metastatic melanoma cells. Increase in proliferation after AD treatment has been also reported in metastatic colon cancer and adenocarcinoma HT-29 cells (40).

Our results suggested that there were no detectable patterns of melanoma cell response to AD that could distinguish CM from UM cells or primary from metastatic melanoma cells. The only difference between CM and UM cells was observed in the zymography assay. In both CM cell lines, unlike UM cells, a band of 130 kDa corresponding to MMP complexes was detected. Moretti et al. (17) have shown that catecholamines (AD and noradrenaline) enhance the motility and invasiveness of melanoma A375 and Hs29-4T cells. These authors have also reported more abundant activation of MMP-2 and MMP-9, which suggested that catecholamines promote the metastatic behaviour of melanoma cells. Our observations partially confirmed that hypothesis, showing stronger activation of MMP-2 than MMP-9 in both CM and UM melanoma cells. It is commonly accepted that the presence and activity of MMPs promote the ability of cancer cells to migrate through the surrounding tissue and cross the basal membrane barrier (36). Moretti et al. (17) reported higher Matrigel invasion rate after AD stimulation of melanoma cells. This was not confirmed by our results from the wound-healing assay. There was not an explicit effect of AD on CM cells migration; however, UM cells seemed to be more susceptible to AD treatment. In both UM cell lines, AD stimulation caused a significantly faster migration of these cells. Simultaneously, we observed a higher migration rate on FN versus plastic, which was consistent with our previously published results regarding migration of melanoma cells (28, 29) and the expression level of integrin α5β1 (29, 41), which is a classical FN receptor. The already described differences in integrin α5β1 expression level between CM and UM cells may provide an explanation for the observed differences in their migration on FN. Slower migration on FN than plastic observed in 92-1 cells may be attributed to already reported low integrin α5β1 expression level.

On the other hand, interesting observations have been made in breast cancer cells that have enabled to identify the dual mechanism of ADR2B activation (42). It has been demonstrated that low concentration of agonist (isoproterenol) increases adhesion of breast cancer cells, whereas higher doses lead to decreased proliferation of these cells. The different activation of signalling pathways has been suggested in this model as an explanation for the observed phenomenon. The authors have suggested that the agonist in low concentration binds preferentially to ADR2B population localised in raft microdomains, therefore activating a dedicated Gs/cAMP/EPAC signalling pathway, which, finally, leads to adhesion. In case of high dose of agonist, activation of receptor population localized outside the rafts and Gs/cAMP/PKA signalling pathways activation has been observed (42). These dualistic mechanisms of adrenergic receptor action may serve as an additional explanation for our inconsistent observations regarding melanoma cell migration and proliferation after AD treatment. This hypothesis, however, needs further investigation.

Taken together, our results demonstrated that, in case of UM cells, their response to AD treatment was coherent and both cell lines showed higher migration rate in the presence of AD. It is of note that there was no effect on UM cell proliferation. These observations might be related to the higher ADR2B expression level in UM cells in comparison to CM
cells. In case of CM cells, we showed that AD did not stimulate but rather inhibited A375 cell migration on FN contradicting the findings of Moretti et al. (17) showing accelerated invasion after AD stimulation of A375 cells. In addition, the primary CM FM-55-P cells did not show any changes in migration rate in the presence of 10 μM AD. These results are in line with the observed low ADR2B mRNA and ADR2B expression levels in FM-55-P cells, as well as lack of changes in their MMPs’ activity after AD stimulation.

Investigation on ADR2B expression and migration of malignant melanoma A375 cells on FN showed more consistent response to AD treatment than those for the primary melanomas that might lead to the final conclusion that origin and/or higher tumourigenic capacity of A375 cells render them less sensitive to AD stimulation. However, it remains unclear whether these differences are the effect of the different origin of UM and CM cells or, rather, cell line-dependent. To our knowledge, this is the first-ever analysis of the effect of AD
on UM cells. For a true understanding of the role of adrenergic stimulation in melanoma progression, more detailed analyses of the response of CM and UM cells, both primary and metastatic, to AD and noradrenaline are required.

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