1. Introduction

The increasing incidence of skin cancer indicates the importance of understanding the pathogenesis of these diseases. Epidemiological studies have revealed a strong association between skin cancer formation and exposure to sunlight [1]. While the sunlight has a wide spectrum of electromagnetic radiation, from the three components of UV light (UV-A, UV-B, and UV-C) UV-A is biologically less effective, UV-C – although highly effective – is absorbed by the atmosphere, therefore, UV-B has the most severe biological effects [2]. UV light has been implicated in the genesis of several forms of cutaneous malignancies including melanoma, basal cell carcinoma, and squamous cell carcinoma [3]. UV irradiation is assumed to lead to mutations in the DNA [4]. Excessive DNA damage or defective DNA repair mechanisms can lead to cell death or, even worse, to malignant transformation of the cells. Under normal conditions, however, cells with damaged DNA should be recognized and eliminated.

Indeed, various repair and safety mechanisms exist to maintain the integrity of the skin epidermis. For example, if UV-light-induced DNA damage is unreparable, cells containing modified DNA can be eliminated by apoptosis [5]. An important event in the process of UV-B-induced apoptosis is the increase of the intracellular calcium concentration ($[Ca^{2+}]_i$; see e.g. [6]) as the cytosolic Ca^{2+} signal seems to be involved in regulating UV-induced apoptosis in HeLa cells [7].

There are a number of possible ways for a cell to increase $[Ca^{2+}]_i$, including voltage- and ligand-gated calcium channels in the surface and endoplasmic reticulum (ER) membranes. In keratinocytes different purinergic receptors have been implicated to play essential roles in deciding the fate of the cells through regulating proliferation and differentiation. While P2Y receptors seem to control the proliferation of these cells, P2X receptors are likely to be responsible for their differentiation [8]. Among the latter $P2X_7$ receptor is an apoptosis-inducing receptor [9], composed of three identical subunits [10]. In keratinocytes, the $P2Y_2$ receptor takes part in the regulation of migration as a negative regulator [11].

In these experiments the connection between UV-B irradiation and the pattern of the expression of purinergic receptors is described. We demonstrate a clear reduction in $P2X_7$ receptor expression following UV-B irradiation. In line with the altered expression pattern, differences in resting $[Ca^{2+}]_i$ and in the way how cells respond to extracellular ATP was also

recognized. These modifications in purinergic signaling could contribute to an altered apoptotic tendency and thus in part explain the increased probability of survival of malignantly transformed cells.

2. Materials and methods

2.1 Culturing HaCaT keratinocytes

HaCaT keratinocytes [12] were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, Budapest, Hungary) supplemented with 10% fetal calf serum (Sigma), 2 mmol/L L-glutamine, and antibiotics (50 NE/ml penicillin, 50 μg/ml streptomicin and 1,25 μg/ml Fungizon) at 37°C temperature in a 5% CO_2 atmosphere as described in our previous reports [13, 14].

2.2 Irradiating the cells

Keratinocytes were cultured in 10 cm diameter Petri dishes. After media removal and wash with phosphate based saline (PBS; Sigma) 0.5 ml of PBS was put on the cells to prevent the exsiccation of the monolayer culture. For irradiation, a broadband UV-B irradiation was applied using a Philips TL-12 (57.5% of UV output in UV-B, 280–315 nm) in 40 mJ/cm^2 dose as described in our previous reports [15]. Proper UV-B dosage was determined by a UVX Digital Radiometer (UVP Inc., San Gabriel, CA, USA); regularly. Mock treated samples were handled the same way as irradiated ones. Untreated controls cells were taken up at the time of the experiment, while UV-B irradiated and mock treated cells were supplied with 37 °C culture media and were used for experiments at 8, 12, 16, 24, 30, 36 and 48 h as indicated.

2.3 Determination of viable cell numbers

The number of viable cells was determined by measuring the conversion of the tetrazolium salt MTT to formazan (Sigma). Cells were plated in 96-well multititer plates (5000 cells/well density) and were treated with UV-B and, at appropriate times, the cells were incubated with 0.5mg/ml MTT for 2 h, and the concentration of formazan crystals (as the indicator of number of viable cells) was determined using a colorimetric assay according to the manufacturer's protocol. Data were expressed as mean ± SEM.

2.4 Determination of apoptosis

To detect the rate of apoptosis, the Dead Cell Apoptosis Kit with Annexin V FITC and PI (Invitrogen, Carlsbad, CA, USA) was used. Cells were plated in 96 multititer plates with black walls and clear bottoms (5000 cells/well density) after the UV-B irradiation. To detect the total cell number CyQUANT® Cell Proliferation Assays (Invitrogen) was used, according to the manufacturer's protocol. To evoke apoptosis 180 μM ATP was administered to the cells. Solution was changed three times after every 12 hours. The apoptotic cell number was normalized to the total cell number.

2.4 RT-PCR analysis.

For RT-PCR analysis, cells were washed three times with CMF-PBS (calcium magnesium free PBS), scraped, snap-frozen in liquid nitrogen and stored at –70 °C. Total RNA was isolated from cells using Quiagen RNeasy® Micro Kit according to the instructions of the manufacturer (Quiagen, Budapest, Hungary). The assay mixture (20 μL) for reverse transcriptase reaction (Omniscript, Quiagen) contained 500 ng RNA, 0.25 μL RNase inhibitor, 0.25 μL oligo(dT), 1 μL dNTP (200 μM), 1μl M-MLV RT in 1× RT buffer. Amplifications of specific cDNA sequences were performed with specific primers (Integrated DNA Technologies, Coralville, IA, USA) that were designed based on published purinoreceptor nucleotide sequences (for sequences of primer pairs, see Supplementary Data). PCR reactions were allowed to proceed in a final volume of 50 μL (containing 1 μL forward and reverse primers, 1 μL dNTP [200 μM], and 5 units Promega GoTaq® DNA polymerase in 1× reaction buffer) in a programmable thermocycler (Eppendorf Mastercycle, Netheler, Hinz GmbH, Hamburg, Germany) with the following settings: 2 min at 95 °C for initial denaturation followed by repeated cycles of denaturation at 94 °C for 1 min, primer annealing for 60 sec at an optimized temperature, and extension at 72 °C for 1 min 30 sec. After the final cycle, further extension was allowed to proceed for another 10 min at 72 °C. PCR products were analyzed using a 1.5 % agarose gel. The gel was stained with 0.01% ethidium bromide solution for 30 minutes, and observed in UV light. Image J program (Wayne Rasband, National Institutes of Health, USA) was used for densitometry analysis. Together with the purinoreceptors GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression was also determined and used as internal control.

2.5 Western blot analysis

To determine the expression of different purinoreceptors in cultured cells, the Western blot technique was applied [16]. Cells in cultures were washed with ice-cold PBS, harvested in homogenization buffer (in mmol/L, 20 TRIS-Cl, 5 EGTA, 1 4-(2-aminoethyl) benzenesulphonyl fluoride, 0,02 leupeptin, pH 7.4; all from Sigma) and disrupted by sonication on ice. Protein content of samples was measured by a modified bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Total cell lysates were mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 10 min at 100 °C. The samples were subjected to SDS-PAGE (8% gels were loaded with 40–50 μg protein per lane) and transferred to nitrocellulose membranes (BioRad, Wien, Austria). Membranes were then blocked with 5% dry milk in PBS and probed with the appropriate, primary antibodies overnight (see Supplementary Data). Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; BioRad) were used as secondary antibodies, and the immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) western blotting detection kit (Amersham, Little Chalfont, England). Image J program was used for densitometry analysis

2.6 Measuring the changes in intracellular calcium concentration

Changes in $[Ca^{2+}]_i$ were monitored using Fura-2, as described in our earlier reports [17, 18]. In brief, isolated cells were placed on a coverslip and loaded with 5 μM Fura-2 AM (acetoxy methyl ester) for 60 minutes. Cells were then equilibrated in Tyrode's solution (in mmol/L, 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 Hepes-NaOH, 1 g/L glucose, pH 7.4, all from Sigma) for a half an hour at room temperature. Coverslips with Fura-2-loaded cells were placed on the stage of an inverted fluorescence microscope (Diaphot; Nikon, Tokyo, Japan). The excitation wavelength was altered between 340 and 380 nm by a microcomputer-controlled dual-wavelength monochromator (Deltascan; Photon Technology International, New Brunswick, NJ), whereas the emission was monitored at 510 nm using a photomultiplier at 10 Hz acquisition rate of the ratios. $[Ca^{2+}]_i$ was calculated from the ratio of measured fluorescence intensities (F_{340}/F_{380}) as described [19]. Cells were continuously washed by Tyrode's solution using a background perfusion system, whereas the investigated agents were applied through a local perfusion system, which is positioned in close proximity to the measured cell.

3. Results

In our case a 1 minute long, 40 mJ/cm^2 UV-B irradiation decreased the number of viable cells as assessed using the MTT assay. Figure 1A shows that, as measured at several points from 8 hours up to 2 days after irradiation, the number of viable cell – expressed as the percent of control – decreased substantially. Albeit not yet significant the decrease was already present 8 hours after the irradiation. It became significant after 16 hours and reached more than 50% after 24 hours. Not only did the number of cells decrease but their morphology was also altered as shown on the phase contrast images of the cells (Fig. 1B). Comparing with the non-irradiated cells, the irradiated cells shrank and lost their extensions.

Cells, nevertheless, responded to challenges of extracellular ATP by elevating their intracellular Ca^{2+} level (Fig. 2). We measured the effect of 180 μM ATP on $[Ca^{2+}]_i$ one day after the irradiation by administering it to the close proximity of cells. Figure 2A and B demonstrate that the administration of ATP induced a transient increase in $[Ca^{2+}]_i$ in both control and in irradiated cells bathed in Ca^{2+}-containing solution. Figure 2A and B also demonstrate that in the non-irradiated cells the amplitude of the response to the ATP challenge (the change in $[Ca^{2+}]_i$) was higher than in irradiated cells. In addition, in case of the irradiated cells, after the response to ATP, $[Ca^{2+}]_i$ returned to its resting level slower than in control cells. Figure 2C presents pooled data for control and irradiated cells. Apart from the above mentioned changes, Fig. 2C also demonstrates that the resting calcium level and the maximal rate of rise of the calcium transients were higher in control cells. All these differences were found to be significant. These observations clearly indicate that the purinergic signaling must have been altered in irradiated cells. To explain these alterations the changes in receptor expression was assessed in both RT-PCR and Western blot experiments.

Previous results from our lab identified the $P2X_1$, $P2X_3$, $P2X_7$ and $P2Y_2$ purinergic receptors as the most important on normal HaCaT cells [14], thus in the followings the changes in the expression patterns of these receptors, especially in that of the proapoptotic $P2X_7$ receptor, were put in focus. Figs. 3 and 4 demonstrate that after seeding the expression of these receptors followed a characteristic pattern. With Western blot, again, the control and irradiated cells were compared. Both ionotropic and metabotropic purinoreceptors were examined after 8, 12, 24, 36h of UV-B irradiation (Fig. 3). First the changes in the expression level of the ionotropic

purinoreceptors were checked. Figure 3 demonstrates that except for 8h after irradiation, where the protein levels seemed to be the same, in every other case the $P2X_1$ protein level was less in the irradiated cells as compared to control (mock-treated) cells at the same time. In the case of $P2X_3$ receptor, after the irradiation, an extra band appeared below the $P2X_3$ protein band. This might indicate that UV-B irradiation damaged some of these receptors. Examining the $P2X_7$ protein levels we could detect the characteristic (80 kDa) band for the intact receptor only in the non-irradiated cells. In the irradiated cells we could only detect an approximately 20 kDa band, which could represent the monomeric form of this receptor [10].

Densitometric analysis was carried out on the above mentioned Western blots (Fig. 4). The protein levels of $P2X_1$ receptor were significantly decreased in the irradiated cells 12 and 36 hours after the irradiation (Fig. 4A). The level of the $P2X_3$ receptor was significantly higher than in the control cells 8, 12 and 24 hours after irradiation (Fig. 4B). In case of $P2X_7$ receptor, the densitomertric analysis was separately made from both 80 kDa and 20 kDa bands. In case of the irradiated cells the 80 kDa band of $P2X_7$ receptor could not be found at all (Fig. 4Ca). 12, 24 and 36 hours after the irradiation the 20 kDa bands of $P2X_7$ receptor were significantly higher in irradiated cells compared to control cells (Fig. 4Cb). In case of the $P2Y_2$ receptor 8 hours after the irradiation the UV-B exposed cells had significantly more $P2Y_2$ protein than did the control cells. 24 and 36 hours after the irradiation, the $P2Y_2$ protein level was significantly less in the irradiated as compared to control cells (Fig. 4D).

The mRNA levels of the receptors were checked with RT-PCR (Fig. 5). Except from 24 h after irradiation, where the mRNA levels seemed to be the same in both control and irradiated cells, we detected less $P2X_7$ receptor mRNA after irradiation. RT-PCR also indicated that after irradiation the $P2Y_2$ mRNA levels decreased, and after 36 hours from the irradiation, the mRNA of this receptor disappeared from the UV-B treated cells (Fig 5A). Densitometric analysis for these receptors were made by normalizing the bands to that of GAPDH. Figure 5B demonstrates that the mRNA level of the $P2X_7$ receptor was significantly higher in the control as compared to the irradiated cells 8, 12 and 36 hours after the irradiation. The $P2Y_2$ mRNA level was significantly lower in the irradiated cells as compared to control after 12, 24 and 36 hours of the irradiation (Fig 5C).

As a next step the rate of apoptosis was measured in control and UV-B irradiated cells using established fluorescence methods. Although equal number of cells were plated, the number

of cells at the time of the measurement were different as expected from the results presented in Fig. 1. To correct for this difference the rate of apoptosis was first normalized to cell count. The rate of apoptosis in irradiated cells – in the absence and presence of ATP – was then expressed as percentage of the values obtained for non-irradiated cells (Fig 6). Although already the basal rate of apoptosis (in the absence of ATP) was slightly smaller in UV-B irradiated than in control cells (78±19%, n=8), this difference did not prove to be statistically significant ($p>0.2$). On the other hand, if apoptosis was induced by the addition of ATP into the bathing medium (for both non-irradiated and irradiated cells) the rate of apoptosis was significantly smaller in UV-B irradiated cells as compared to non-irradiated (mock-treated) cells (61±22%, n=8; $p<0.02$), in line with the absence of functional $P2X_7$ receptors on these cells.

4. Discussion

In our experiments, we examined how keratinocytes respond to UV-B irradiation in respect to their purinergic calcium signaling. One minute long, 40 mJ/cm^2 UVB irradiation – corresponding to approximately 2-4 minimal erythemal dose in an intact skin [20, 21] – was used to check the accompanying changes in the expression levels of purinoreceptors, and the changes in the response of the cells to the extracellular application of ATP. A one minute long 40 mJ/cm^2 UVB exposure was selected because longer irradiation times resulted in the death of essentially every cell. Similar irradiation time and light intensity was used in our previous measurements concerning gene expression [15], therefore these data are directly comparable to those results. Importantly though, even this low energy exposure to UV-B light decreased the viable cell number significantly as compared to control cells.

In the events of programmed cell death one of the first steps is the increase in the intracellular calcium concentration of the cell. Several studies have linked the activation of purinoreceptors especially that of the $P2X_7$ receptor [9], [22] to the initiation of apoptotic cell death. *In vivo* these purinoreceptors may be activated by ATP coming from other dead cells, or physiologically released from nerves [23], uroepithel [24] and endothel [25]. In the human squamosus cell carcinoma line A431 micromolar concentrations of ATP were mitogenic [26] and increased intracellular calcium levels [27]. ATP has also been shown to activate cell proliferation in MCF-7 breast cancer cells [28] and in human ovarian tumor cells [29]. Importantly tough high

concentrations of ATP and BzATP (specific agonist of the $P2X_7$ receptor) caused a significant decrease in A431 cell number [30]. In our experiments, after irradiation, the $P2X_7$ receptors were diminished in the cells, and we detected less $P2X_7$ receptor mRNA. This might indicate that cells which were damaged to some extent but survived the irradiation were not as capable of initiating apoptotic cell death. This in turn could give rise to a population of cells with damaged DNA but low apoptotic capabilities thus enabling malignantly transformed cells to persist. It should be noted, however, that while DNA damage in the skin is commonly described in association with UV-B irradiation [31] the direct degradation of proteins following exposure to UV-B light is far less frequently observed [32].

Other P2X and P2Y receptors also have important functions in regulating the proliferation and differentiation of cells. The metabotropic $P2Y_1$ and $P2Y_2$ receptors were shown to control the proliferation of keratinocytes [8]. When a skin cancer evolves from healthy keratinocytes, the pattern of these purinoreceptors may change. We found that a 1 minute long 40 mJ/cm^2 UV-B irradiation decreased the amount of both $P2X_1$ and $P2Y_2$ receptors, while essentially destroyed the $P2X_7$ receptors. In particular, $P2Y_2$ receptors have been associated with promoting proliferation in both normal human keratinocytes and in HaCaT cells [33]. In our experiments, we demonstrated that the viable cell number was decreased after the 1 minute long 40 mJ/cm^2 UV-B irradiation, in which the decreased amount of $P2Y_2$ receptors might also have played a role. Indeed, the experiments revealed that the mRNA of the $P2Y_2$ receptor was less in the UV-B treated than in control cells, and 36 and 48 hours after the irradiation the mRNA of this receptor disappeared from the irradiated cells. Although 8 hours after the irradiation the protein level of the $P2Y_2$ receptor was higher in the UV-B treated than in the control cells, the amount of this receptor was decreased in the UV-B treated cells at every other point in time. By loosing much of these receptors, cells could lose the control over the proliferation, differentiation or both, and thus be shifted towards a malignant transformation.

In line with the altered expression of purinoreceptors, differences in the calcium transients obtained in response to challenges with ATP were also observed. The amplitude and the rate of rise of these calcium transients were lower in the irradiated cells, which may reflect the lower amount of purinergic receptors on these cells. The latter might also be caused by the shift from mostly ionotropic (P2X) to partly metabotropic (P2Y) response in the irradiated cells. For $[Ca^{2+}]_i$ to return to its resting level took longer times in the irradiated cells. This could indicate that other

proteins, for example the calcium pump was also damaged by UV-B irradiation. This notion should, however, be taken with caution since resting $[Ca^{2+}]_i$ was lower in irradiated than in control cells.

Acknowledgements

The authors wish to thank Vargáné Kiss Ibolya for the excellent technical assistance. This work was supported by grants from the Hungarian Science Fund (OTKA NK78398 and K68401), ETT (186/09), and TÁMOP 4.2.2.-08/1-2008-0019.

References

1. S.D. Worswick, M. Cockburn, D. Peng, Measurement of ultraviolet exposure in epidemiological studies of skin and skin cancers, Photochem Photobiol, 84 (2008) 1462-1472
2. W. Ambach, M. Blumthaler, Biological effectiveness of solar UV radiation in humans, Experientia, 49 (1993) 747-753
3. C. Perlis, M. Herlyn, Recent advances in melanoma biology, Oncologist 9 (2004) 182-187
4. D. Bettega, P. Calzolari, F. Belloni, F. Di Lena, S. Genchi, M. Lupi, P. Massariello, S. Orsini, L. Tallone, D. Tomasoni, P. Ubezio, J.L. Redpath, Solar UV radiation: differential effectiveness of UVB subcomponents in causing cell death, micronucleus induction and delayed expression of heritable damage in human hybrid cells, Int J Radiat Biol. 77 (2001) 963-970
5. P. Erb, J. Ji, E. Kump, A. Mielgo, M. Wernli, Apoptosis and pathogenesis of melanoma and nonmelanoma skin cancer, Adv Exp Med Biol 624 (2008) 283-295
6. F.M. Lyng, C.B. Seymour, C. Mothersill, Early events in the apoptotic cascade initiated in cells treated with medium from the progeny of irradiated cells, Radiat Prot Dosimetry 99 (2002) 169-172
7. Y. Pu, D.C. Chang, Cytosolic Ca2+ signal is involved in regulating UV-induced apoptosis in HeLa cells, Biochem Biophys Res Commun, 282 (2001) 84-89

8. A.V. Greig, C. Linge, G. Terenghi, D.A. McGrouther, G. Burnstock, Purinergic receptors are part of a functional signaling system for proliferation and differentiation of human epidermal keratinocytes, J Invest Dermatol, 120 (2003) 1007-1015

9. A. Surprenant, F. Rassendren, E. Kawashima, R.A. North, G. Buell, The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$), Science 272 (1996) 735-738

10. D. Becker, R. Woltersdorf, W. Boldt, S. Schmitz, U. Braam, G. Schmalzing, F. Markwardt, The $P2X_7$ carboxyl tail is a regulatory module of $P2X_7$ receptor channel activity, J Biol Chem, 283 (2008) 25725-25734

11. S. Taboubi, F. Garrouste, F. Parat, G. Pommier, E. Faure, S. Monferran, H. Kovacic, M. Lehmann, Gq-coupled purinergic receptors inhibit insulin-like growth factor-I/phosphoinositide 3-kinase pathway-dependent keratinocyte migration, Mol Biol Cell, 21 (2010) 946-955

12. P. Boukamp, R.T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham, N.E. Fusenig, Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line, J Cell Biol, 106 (1988) 761-771

13. M. Gönczi, N. Szentandrássy, L. Fülöp, A. Telek, G.P. Szigeti, J. Magyar, T. Bíró, P.P. Nánási, L. Csernoch, Hypotonic stress influence the membrane potential and alter the proliferation of keratinocytes in vitro, Exp Dermatol, 16 (2007) :302-310

14. M. Gönczi, A. Telek, G. Czifra, A. Balogh, P.M. Blumberg, T. Bíró, L. Csernoch, Altered calcium handling following the recombinant overexpression of protein kinase C isoforms in HaCaT cells, Exp Dermatol, 17 (2008) 584-591

15. A. Balogh, G. Paragh Jr, A. Juhász, T. Köbling, D. Törocsik, E. Mikó, V. Varga, G. Emri, I. Horkay, B. Scholtz, E. Remenyik, Reference genes for quantitative real time PCR in UVB irradiated keratinocytes, J Photochem Photobiol B, 93 (2008) 133-139

16. H. Papp, G. Czifra, J. Lázár, M. Gönczi, L. Csernoch, L. Kovács, T. Bíro, Protein kinase C isoenzymes regulate proliferation and high cell density-mediated differentiation in HaCaT keratinocytes, Exp Dermatol, 12 (2003) 811-824

17. H. Szappanos, J. Cseri, T. Deli, L. Kovács, L. Csernoch, Determination of depolarisation- and agonist-evoked calcium fluxes on skeletal muscle cells in primary culture, J Biochem Biophys Methods, 59 (2004) 89-101

18. A. Szücs, H. Szappanos, A. Tóth, Z. Farkas, G. Panyi, L. Csernoch, I. Sziklai, Differential expression of purinergic receptor subtypes in the outer hair cells of the guinea pig, Hear Res. 196 (2004) :2-7

19. G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca2+ indicators with greatly improved fluorescence properties, J Biol Chem 260 (1985) 3440-3450

20. , J. Shorrocks, N.D. Paul, T.J. McMillan, The dose rate of UVA treatment influences the cellular response of hacat keratinocytes, J Invest Dermatol 128 (2008) 685–693

21. Y. Furusawa, L. E. Quintern, H. Holtschmidt, P. Koepke, M. Saito, Determination of erythema-effective solar radiation in Japan and Germany with a spore monolayer film optimized for the detection of UVB and UVA - results of a field campaign, Appl Microbiol Biotechnol 50 (1998) 597-603

22. J.N. Tran, A. Pupovac, R.M. Taylor, J.S. Wiley, S.N. Byrne, R. Sluyter, Murine epidermal Langerhans cells and keratinocytes express functional $P2X_7$ receptors, Exp Dermatol 19 (2010) e151-157

23. B. Sperlágh, A. Kittel, A. Lajtha, E.S. Vizi, ATP acts as fast neurotransmitter in rat habenula: neurochemical and enzymecytochemical evidence, Neuroscience 66 (1995) 915-920

24. D.R. Ferguson, Urothelial function, BJU Int 84 (1999) 235-242

25. P. Bodin, G. Burnstock, Increased release of ATP from endothelial cells during acute inflammation, Inflamm Res 47 (1998) 351-354

26. N. Huang, D.J. Wang, L.A. Heppel, Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors, Proc Natl Acad Sci U S A 86 (1989) 7904-7908

27. F.A. Gonzalez, D.J. Gross, L.A. Heppel, W.W. Webb, Studies on the increase in cytosolic free calcium induced by epidermal growth factor, serum, and nucleotides in individual A431 cells, J Cell Physiol 135 (1988) 269-276

28. C.J. Dixon, W.B. Bowler, P. Fleetwood, A.F. Ginty, J.A. Gallagher, J.A. Carron, Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors, Br J Cancer. 75 (1997) 34-39

29. L.D. Popper, S. Batra, Calcium mobilization and cell proliferation activated by extracellular ATP in human ovarian tumour cells, Cell Calcium 14 (1993) 209-218

30. A.V. Greig, C. Linge, V. Healy, P. Lim, E. Clayton, M.H. Rustin, D.A. McGrouther, G. Burnstock, Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells, J Invest Dermatol 121 (2003) 315-327

31. F.R. de Gruijl, H. Rebel, Early events in UV carcinogenesis - DNA damage, target cells and mutant p53 foci, Photochem Photobiol. 84 (2008) 382-387

32. M.A.K. Jansen, V. Gaba, B.M. Greenberg, A.K. Mattoo, M. Edelman, Low threshold levels of ultraviolet-B in a background of photosynthetically active radiation trigger rapid degradation of the D2 protein of photosystem-II, The Plant Journal 9 (1996) 693-699

33. H.E. Burrell, W.B. Bowler, J.A. Gallagher, G.R. Sharpe, Human keratinocytes express multiple P2Y-receptors: evidence for functional $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors, J Invest Dermatol 120 (2003) 440-447

Figure legends

Fig. 1. Examination of cell viability

Effect of UV-B irradiation (1 min, 40 mJ/cm^2) on viable cell number and cell morphology. (A) HaCaT cell viability was measured at the times indicated using the MTT assay and expressed as normalized to the non-irradiated (mock) control. Here and in all subsequent figures asterisks indicate significant (* $p<0.05$; ** $p<0.01$) differences as compared to control. (B) Phase contrast images of non-irradiated (upper panel) and irradiated cells 24 h after irradiation. Note the changes in the cell number and especially in morphology. Inserts show enlarged images of selected cells to further the comparison. Scale bars represent 50 μm.

Fig. 2. ATP-induced Ca^{2+} transients

ATP-induced changes in $[Ca^{2+}]_i$ in control (untreated) (A) and in irradiated cells (B) bathed in Ca^{2+}-containing extracellular solution. Note, that in the non-irradiated cells the amplitude of the ATP-induced Ca^{2+} transient (the change in $[Ca^{2+}]_i$) was higher than in irradiated cells. In addition, in case of the irradiated cells $[Ca^{2+}]_i$ returned to its resting level slower than in control cells. (C) Pooled data of the characteristic parameters of ATP-induced Ca^{2+} transients for control and irradiated cells.

Fig. 3. Western blot experiments

Protein levels of four purinoreceptors - $P2X_1$, $P2X_3$, $P2X_7$ and $P2Y_2$ – characteristic for HaCaT cells as detected by Western blot 8, 12, 24 and 36 hours after UV-B (1 min, 40 mJ/cm^2) irradiation. UV-B irradiation usually decreased the protein level as compared to the control (mock-treated). Note the marked degradation in the $P2X_7$ protein level of HaCaT cells. Except for 8 h after irradiation, we could only detect an approximately 20 kDa band in the UV-B irradiated samples, whereas in control cells we could see the 80 kDa band which is characteristic for the $P2X_7$ receptor.

Fig. 4. Densitometric analysis of Western blots

Densitometric analysis of Western blots presented in Fig. 3 (panel A – $P2X_1$; panel B – $P2X_3$; panel C – $P2X_7$; panel D – $P2Y_2$). Black columns represent the non-irradiated cells (mock-

treated), while grey columns show the UV-B irradiated samples. Note that the 80 and 20 kDa bands for the $P2X_7$ receptor are presented as separate graphs (panel Ca for 80 kDa protein and panel Cb for 20 kDa fragment, respectively).

Fig 5. mRNA measurements

mRNA levels of $P2X_7$ and $P2Y_2$ receptors were measured in control (mock-treated) and irradiated cells (A). GAPDH was used as control, and values were normalized to the GAPDH data. Panel B and C present pooled data for the mRNA of $P2X_7$ and $P2Y_2$ receptors, respectively, obtained from the densitometric analysis of experiments similar to that in Fig. 4.

Fig. 6. Apoptosis assay

Rate of apoptosis was measured in control (mock-treated) and UV-B irradiated cells and normalized to total cell count. Values from irradiated samples were expressed as percentage of those obtained under control conditions.