Abstract. UV light excites aromatic residues, causing these to disrupt nearby disulphide bridges. The EGF receptor is rich in aromatic residues near the disulphide bridges. Herein we show that laser-pulsed UV illumination of two different skin-derived cancer cell lines i.e. Cal-39 and A431, which both overexpress the EGF receptor, leads to arrest of the EGFR signaling pathway. The phosphorylation status of the receptor and the level of phosphorylated downstream signaling molecules i.e. AKT and the mitogen activated protein kinases (MAPKs) ERK1 and 2 is detected by Western blotting using phosphospecific antibodies. There was a threshold level, below which the receptor could not be blocked. In addition, illumination caused the cells to upregulate the cyclin-dependent kinase inhibitor p21 WAF1, irrespective of the p53 status. Since the EGF receptor is often overexpressed in cancers and other proliferative skin disorders, it might be possible to significantly reduce the proliferative potential of these cells making them good targets for laser-pulsed UV light treatment.

Introduction

The epidermal growth factor receptor (EGFR), also known as HER1/Erb-B1 belongs to the ErbB family of receptor tyrosine kinases (RTKs) (1,2). Binding of ligands such as EGF and TGF, leads to homo- and heterodimerization of the receptors (3). Dimerization in the case of EGFR leads to autophosphorylation of specific tyrosine residues in the intracellular tyrosine kinase domain. These phosphorylated tyrosine residues serve as docking sites for other kinases e.g. phosphatidylinositol-3-kinase (PI3K) and adaptor proteins e.g. Shc and Grb2. This in turn initiates cascades of intracellular signaling pathways including the PI3K-AKT pathway and the Ras-Raf-MAPK pathway (2,3). Activation of the ErB receptor family triggers a number of different responses including mitogenesis, apoptosis, cellular motility, angiogenesis and differentiation (2). Activation of the MAPK and PI3K/AKT signaling pathways leads to the expression of mitogenic factors, which can induce the malignant conversion of keratinocytes, tumor progression and invasive potential (4-9). This is supported by the finding that the EGF receptor is overexpressed or subject to uncontrolled signaling in a number of solid tumors (10-21) and is often associated with poor prognosis and advanced disease. The current strategy in targeting the ErbB receptors includes monoclonal antibodies and small-molecule kinase inhibitors, which inhibit autophosphorylation and downstream signaling (22).

The spatial proximity between aromatic residues and disulphide bridges in proteins has been preserved during molecular evolution (23,24). UV illumination of aromatic residues in proteins leads to the disruption of nearby disulphide bridges, leading to free reactive thiol groups, radicals and ions (25-28, Neves-Petersen et al., unpublished data). Interestingly, the EGF receptor is rich in these amino acids known to be involved in photophysical and photochemical reactions triggered by UV light.

Herein we show that LP-UV treatment of two skin-derived tumor cell lines, i.e. A431 and Cal-39 leads to the inhibition of the EGF receptor and key downstream molecules such as AKT1 and ERK1/2 involved in the RTK-catalyzed signaling cascade. Our results show a potential for treatment of skin diseases associated with increased proliferation relating to the EGF receptor e.g. warts (29), condylomas (30,31), psoriasis (32,33) and skin cancer.

Materials and methods

Cell culture. A431 cells (human epidermoid carcinoma cells) were maintained in RPMI medium (Gibco BRL) supplemented with 10% fetal bovine serum (Bio Whittaker Europe). Cal-39 cells (human vulva squamous cell carcinoma cells) were maintained in DMEM medium (Gibco BRL) supplemented with 20% fetal bovine serum, 0.5 nM hydrocortisone (Clonetics) and 0.01 μg/ml EGF (Calbiochem). Both cell
lines were obtained from DSMZ, Braunschweig, Germany and kept at 37°C in a humidified atmosphere and 5% CO₂. For the experiments, 80-85% confluent cells were serum-starved for 18 h prior to light illumination.

Illumination. Illumination was carried out with femtosecond lasting laser pulses at 280 nm. The pulses were generated by sending the output from a Spectra Physics Tsunami laser (<100fs pulse duration, 12 nm FWHM, 80 MHz repetition rate, λ=840 nm, Tsunami 3960, Spectra Physics, Mountain View, CA) pumped by a high power (5 W at 532 nm) solid state laser Millennia V, Spectra Physics) through a pulse picker, which decreased the pulse repetition rate to 8 MHz. The fundamental pulse was mixed with its second harmonic (420 nm) in a frequency doubler/tripler (GWU; Spectra Physics) to generate a pulse at 280 nm. The power of the 280 nm light after GWU was 0.273 mW. The pulse was expanded prior to sample illumination with a diffusive lens in order to illuminate as large an area as possible (half a petri dish, d=35 mm). Each halfplate was illuminated at the indicated times. For activation of the EGFR pathway the cells were incubated with 100 ng/ml EGF (Calbiochem) for 5 min at 37°C either prior to or after illumination.

Protein extraction and Western blotting. Cells were washed once in ice-cold PBS and scraped in 50 μl lysis buffer [50 mM Tris–HCl pH 8.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 30 mM NaPPi, 10 mM NaF, 1 mM Na₃VO₄, 100 nM okadaic acid, Complete protease inhibitors (Roche)]. Lysates were incubated on ice for 15 min and cleared by centrifugation (4°C, 12000 x g, 30 min). The supernatant was used for protein determination (34). Protein (75 μg) was separated by 10% SDS-PAGE and the proteins transferred to a PVDF membrane (Bio-Rad) by wetblotting in 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% MeOH. The membrane was blocked in blocking buffer (0.2% casein, 0.1% Tween-20 in PBS) for 1 h and incubated with either monoclonal anti-AKT (BD Transduction Laboratories), anti-PARP (Pharmingen), anti-β-actin (Sigma) or polyclonal anti-Phospho-AKT (P-Thr308) or ERK1 (P-Thr202/p-Tyr204) (Cell Signaling), or anti-ERK1/2 (p-Thr202/p44) (Cell Signaling), anti-Phospho-ERK1/2 (P-p42/p44 (Thr202/Tyr204) (Cell Signaling), anti-EGFR (Santa Cruz), anti-Phospho-EGFR (Tyr1173) (Santa Cruz). After washing in blocking buffer the membranes were incubated with either a secondary goat anti-mouse antibody, goat anti-rabbit or sheep anti-goat coupled to alkaline phosphatase (Jackson immunoresearch laboratories) for 1 h. Visualization was done with CDP-star (Tropix) according to the manufacturer’s instructions.

Results

Laser-pulsed UV illumination blocks EGF receptor signaling above a certain power/threshold level in A431 cells. The human skin cancer cell line A431, which overexpress the EGF receptor (>1.5 million receptors per cell (35)) was used to investigate whether laser-pulsed UV illumination could block EGF receptor signaling. Interestingly, the EGF receptor contains aromatic residues in close proximity to S-S bridges making it a likely candidate for light-induced immobilization.

To assess the time required to inactivate the EGF receptor with UV light, a time course experiment was performed in A431 cells (Fig. 1). Cells were serum-starved prior to treatment and the cells were either illuminated at different time-points and then incubated with EGF or first treated with EGF followed by different UV light illumination times. The blockage in EGF receptor signaling was detected by Western blotting using phospho-specific antibodies against the EGFR receptor and the downstream signaling molecules AKT1 and ERK1/2. As seen in Fig. 1, there exists a power/threshold level above which it is possible to inactivate the EGFR receptor, as seen by no phosphorylation of the EGFR receptor and the downstream signaling molecules AKT and ERK1 and 2 (lanes 7-10), whereas below this threshold (lanes 1-6) no inactivation could be detected by Western blotting. Detection of total EGFR, AKT1 and ERK1/2 protein was used to ensure equal loading. The results show that illumination times for more than 30 min attenuate the EGFR signaling pathways.

Laser-pulsed UV immobilization also blocks EGF receptor signaling in Cal-39 cells. To see if the observed blockage of EGFR signaling in A431 cells also is found in other cell lines, illumination experiments were performed in another human skin cancer cell line, i.e. Cal-39, which expresses lower levels of the EGF receptor (Fig. 2 left and right part). Again Western blot detection of phosphorylated EGFR, AKT1 and ERK1/2 was used to assess the effect of the UV illumination.

Fig. 2 left part shows the results from the human squamous cell line A431. The cells were serum-starved prior to incubation with EGF and illumination. As expected, serum-starved cells (lane 1; control) showed no phosphorylation of either EGFR, AKT1 or ERK1/2, whereas incubation of cells with EGF led to activation of the EGFR signaling cascade assessed by phosphorylation of both the EGF receptor, and the two downstream effectors AKT and ERK1/2 (lane 2; EGF). EGF
incubation prior to illumination did not change its ability to activate the phosphorylation cascade (lane 4; EGF, UV), whereas UV illumination prior to EGF treatment prevented activation of the EGFR, AKT1 and ERK1/2 (lane 4; UV, EGF), hence there is no effect of EGF once the cells have been illuminated. Western blot detection of total EGFR, AKT and ERK1/2 proteins show that the protein level is not affected by illumination.

The results using Cal-39 cells were the same (Fig. 2 right part) as for A431 cells. Illumination prior to EGF incubation prevented activation of the EGFR signaling cascade, whereas illumination after incubation with EGF had no effect on the activation of the signaling pathway. These results support the hypothesis that laser-pulsed UV can impact the EGF receptor and prevents its activation by ligand binding.

Laser-pulsed UV illumination upregulates p21WAF1 irrespective of the p53 status. Treatment of cells with monoclonal antibodies against the EGF receptor or tyrosine kinase inhibitors have been shown to upregulate the cyclin-dependent kinase inhibitor, p21WAF1 (36-40).

To test if p21WAF1 was upregulated in response to laser-pulsed UV illumination, both serum-starved Cal-39 and A431 cells were illuminated and returned to full medium and incubated for 24 h. The levels of p21WAF1 in illuminated cells in comparison to not illuminated cells were detected by Western blotting using an anti-p21WAF1 specific antibody. The Western blot (Fig. 3) shows that p21 is indeed upregulated in response to UV illumination. p21WAF1 is a p53 responsive gene, hence we tested the expression of p53 in response to illumination. The A431 cells express mutated p53 (41), which is not upregulated in response to illumination, whereas Cal-39 cells express wild-type p53, which is upregulated in response to illumination (Fig. 3). Although p53 is mutated in A431 cells, both cell lines show an upregulation of p21WAF1 in response to illumination.

Discussion

The ErbB family of receptor tyrosine kinases are often found overexpressed in different tumor types, and the overexpression of the receptors is thought to play a role in the pathogenesis of cancer, hence targeting the family members including the EGF receptor is used in cancer therapy. Targeting includes monoclonal antibodies directed against the external moiety of the EGF receptor, which prevents ligand binding and small molecules, which interfere with the intracellular part of the receptor molecule, i.e. its tyrosine kinase activity (42). Herein we describe a new way of inhibiting the EGF receptor using laser-pulsed UV illumination. Laser-pulsed UV illumination targets excitation of aromatic amino
acid residues, leading to disruption of proximal disulphide bridges (25-28). Interestingly, the EGF receptor contains the spatial proximity between disulphide bridges and aromatic residues and in the case of the EGF receptor this disruption prevents downstream signaling from the EGF receptor. As proposed in Fig. 4, the blockage could be through disruption of S-S bridges in the extracellular domain causing the 3-dimensional structure of the receptor to be altered, preventing the binding of ligand. In addition the intracellular domain of the EGF receptor contains a high number of aromatic residues including the phosphorylation-targeted tyrosine residues, thus it is also possible that laser-pulsed UV illumination affects the intracellular tyrosines hence preventing downstream signaling.

Fig. 1 shows that an illumination time/power threshold exists above which laser-pulsed UV light can halt EGFR signaling cascades. Blockage of EGFR signaling is not possible below that threshold. This observation is correlated with the fact that above a certain power/illumination time threshold, proteins lose their native 3D structure and functionality. Importantly, the S-S disruption is a reversible process at low power/illumination times.

We describe the attenuation of EGFR signaling as detected by the phosphorylation status of key downstream molecules i.e. AKT and the mitogen activated protein kinases ERK1 and 2. In response to laser-pulsed UV illumination phosphorylation of AKT and ERK1/2 is not detectable upon activation with EGF.

Several reports exist, describing how UV light can activate the EGFR receptor hence activating the AKT and MAPK pathway (43-50); this is thought to be mediated through: i) increased expression of EGFR ligands (51,52) and ii) inactivation of receptor-associated phosphatases and iii) altered internalization and degradation (44,45,53). These observations are in contrast to our results. The reason for this discrepancy could be found in the illumination power per unit of illuminated area (fluency). In our experiments the total integrated power over a second is significantly less than the average solar UV output but comparing the actual output during a pulse event we have 1000-fold higher intensity during the pulse event.

Similarly to the results obtained here concerning the up-regulation of p21<sub>WAF1</sub> mRNA in response to illumination are results in different tumor cell lines treated with inhibitors of the EGF receptor. Blockage of the EGFR receptor by treating with the inhibitor Gefitinib (Iressa), leads to an upregulation of p21<sub>WAF1</sub> mRNA in a bladder adenocarcinoma cell line (36)

The upregulation of p21<sub>WAF1</sub>, a p53 responsive gene was observed in different tumor cell lines treated with inhibitors of the EGF receptor. Blockage of the EGFR receptor by treating with the inhibitor Gefitinib (Iressa), leads to an upregulation of p21<sub>WAF1</sub> mRNA in a bladder adenocarcinoma cell line (36).

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