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Lovastatin protects keratinocytes from DNA damage-related pro-apoptotic stress responses stimulated by anticancer therapeutics

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ABSTRACT

Background: Oral mucositis (OM) is a relevant adverse effect of anticancer therapy involving ionizing radiation (IR) and doxorubicin (Doxo). Because DNA damage of keratinocytes is causative for the pathogenesis of OM, we aim to identify pharmacological measures for geno- and cytoprotection of keratinocytes.
Methods: We investigated the influence of the lipid-lowering drug lovastatin on cell death, proliferation and DNA damage response (DDR) mechanisms of human keratinocytes following treatment with IR and Doxo.
Results: Lovastatin protected keratinocytes from the cytotoxic and genotoxic effects of IR and Doxo as shown by a diminished induction of apoptosis as well as a reduced formation and slightly improved repair of DNA damage following Doxo and IR treatment, respectively. Lovastatin selectively blocked the activation of Chk1 and ATR kinases following treatment with IR, Doxo and the ribonucleotide reductase inhibitor hydroxyurea, indicating that the statin antagonizes ATR/Chk1-regulated replicative stress responses. Part of the cytoprotective activity of lovastatin seems to rest on a delayed entry of lovastatin treated cells into S-phase. Yet, because the statin also protected non-proliferating keratinocytes from IR- and Doxo-induced cytotoxicity, cell cycle independent protective mechanisms are involved, too.
Conclusions: Lovastatin attenuates pro-toxic DNA damage-related responses of keratinocytes stimulated by OM-inducing anticancer therapeutics. The data encourage forthcoming in vivo and clinical studies addressing the usefulness of statins in the prevention of OM.

1. Introduction

Apart from their favored cytotoxic effects on malignant cells anticancer drugs and ionizing radiation also cause adverse normal tissue damage. Oral mucositis is a frequent acute side-effect of anticancer therapeutics, which is characterized by inflammation of the oral mucous membranes and keratinocyte growth inhibition resulting in erythematous and ulcerative lesions [1,2]. The majority of the head and neck cancer patients undergoing local radiotherapy and 40–80% of patients that receive systemic chemotherapy comprising anthracyclines, 5-fluorouracil or cisplatin develop this painful condition [3,4]. Oral mucositis leads to an increased risk of microbial infections and often entails parenteral nutrition, long-lasting intake of analgetics and extended hospitalization periods. This causes substantial costs to the health system and, most important, also has a considerable impact on the patient's quality of life. Furthermore, dose reduction might become necessary, which limits the efficacy of the antitumor therapy [5,6]. Although numerous options for therapeutic interventions have been investigated, keratinocyte growth factor-1 (palifermin) is the only effective anti-mucositis drug, which has been approved by the US Food and Drug Administration (US-FDA) and the European Medicines Agency (EMA) as specific anti-mucositis agent [7]. Notably yet, as a growth factor, palifermin does not primarily prevent the development of oral mucositis but rather delays its onset and accelerates the healing process.

Oral mucositis develops as a consequence of the geno- and cytotoxic properties of radio- and chemotherapy. In the initiation phase, inhibition of proliferation and cell death of basal epithelial cells (keratinocytes) are the key events that are believed to be due to the induction of DNA damage [8]. Apart from the primary DNA damage, radio- and chemotherapy-associated generation of reactive oxygen species (ROS) further contributes to the activation of detrimental downstream signaling pathways [1]. The complex sequence of molecular and cellular events involves the activation of a large number of transcription factors, including nuclear factor-kappaB (NF-κB). NF-κB stimulates the production of pro-

Abbreviations: 53BP1, tumor suppressor p53 binding protein 1; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Chk, checkpoint kinase; DSB, DNA double-strand break; DDR, DNA damage response; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; Doxo, doxorubicin; 5-FU, 5-fluorouracil; γH2AX, Ser139 phosphorylated histone H2AX; HU, hydroxyurea; IR, ionizing radiation; JNK, c-Jun N-terminal kinase; Kap1, KRAB-associated protein 1; Lova, HMG-CoA-reductase inhibitor lovastatin; NF-κB, nuclear factor kappaB; PARP, Poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; RPA, replication protein A; SAPK, stress activated protein kinase; SSB, DNA single-strand break.

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inflammatory cytokines, for instance tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β) and interleukin-6 (IL-6), which trigger and amplify inflammatory processes, and, moreover, determine the outcome of anticancer-drug exposure by affecting death and survival pathways [9,10]. Furthermore, different types of protein kinases, including c-Jun N-terminal kinases/stress activated protein kinases (JNK/SAPK) and p38 MAP kinase are activated following exposure to radiation or chemotherapeutics and also influence cell survival [11–13]. Bearing in mind that DNA damage to keratinocytes is considered as a primary trigger of oral mucositis [8] and the resulting DNA damage response (DDR) determines DNA repair, survival and death [14,15], protection of keratinocytes from genotoxic insults evoked by anticancer drugs and/or pharmacological modulation of the DDR is considered as promising preventive strategies.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are effective and frequently-used drugs in the treatment of hypercholesterolemia. Apart from their cholesterol lowering activity statins also have pleiotropic, cholesterol-independent effects [16,17]. These are based on a depletion of isoprenoid moieties that are required for post-translational modifications of key signaling proteins (i.e. Ras- and Rho-GTPases) [18,19]. As a consequence, statins influence several cellular functions related to cell homeostasis, proliferation, differentiation as well as survival and death. Among others, statins trigger apoptosis in various tumor cells of different origin [20,21] and counteract metastatic processes in vivo [22,23]. Moreover, statins are reported to radiosensitize tumor cells, i.e. lung cancer cells and lymphoma cells [24,25], and increase the sensitivity of tumor cells to a variety of anticancer drugs including doxorubicin [21,26,27]. Regarding normal cells, statins exhibit multiple inhibitory effects on stress responses stimulated by anticancer therapeutics. For instance, lovastatin impacts cisplatin- and doxorubicin-induced activation of JNK/SAPK [28,29] as well as stimulation of NF-κB following ionizing radiation (IR) and doxorubicin treatment [30,31]. In line with this, statins protect human umbilical vein endothelial cells (HUVEC) from the cytotoxic effects of doxorubicin [28] and radiation damage in vitro [32,33]. Noteworthy, statins are also radioprotective for normal colon cells in vivo [34,35]. Additionally, it has been shown that statins accelerate the repair of oxidative DNA damage in vascular smooth muscle cells via stimulating the NBS pathway [36]. Based on these data, we hypothesized that pharmacological modulation of genotoxin-induced stress responses by statins might also mitigate anticancer drug-induced keratinocyte damage that triggers mucositis. To substantiate this hypothesis, we investigated the molecular effects of lovastatin on stress responses of keratinocyte cells (HaCaT), which are frequently used as in vitro model for mucositis research [37–39], following treatment with ionizing radiation and doxorubicin as prototypical mucositis-inducing anticancer therapeutics.

2. Materials and methods

2.1. Materials

The 3-hydroxy-3-methylglutaryl CoA reductase inhibitor lovastatin, hydroxyurea and 5-fluorouracil were purchased from Sigma (Steinheim, Germany). Doxorubicin was from Cellpharm (Bad Vilbel, Germany). Cisplatin originated from TEVA (Ulm, Germany). Antibodies directed against caspase 3, activated caspase 7, Ser15 phosphorylated protein 53 (pp53), Ser345 phosphorylated checkpoint kinase-1 (pChk1), Ser1981 phosphorylated ataxia telangiectasia mutated (pATM), Ser428 phosphorylated ataxia telangiectasia and Rad-3 related (pATR), 53BP1 and Talin-1 were obtained from Cell Signaling (Beverly, MA, USA). Antibody detecting Thr1989 phosphorylated ATR originates from GeneTex (Irvine, CA, USA). Anti-Ser4/Ser8 phosphorylated replication protein A (pRPA32) and anti-Ser824 phosphorylated KRAB-associated protein-1 (pKap1) antibody originated from Bethyl Laboratories Inc. Anti-Poly(ADP-ribose)-polymerase 1 (PARP-1) and anti-β-actin antibodies were from Santa Cruz (CA, USA). Anti-Ser139 phosphorylated histone 2AX (γH2AX) and anti-protein phosphatase 2A, subunit C (PP2A) antibody were received from Millipore (Billerica, MA, USA) and anti-Thr68 phosphorylated checkpoint kinase-2 (pChk2) from Abcam (Cambridge, MA, USA). Ser10 phosphorylated histone H3 (pH3) antibody, cyclin D1 antibody and cyclin D3 antibody are from Thermo Fisher Scientific (Waltham, MA, USA). Anti-cyclin A, anti-cyclin B1 and anti-cyclin E antibodies were purchased from BD Biosciences (San Jose, CA, USA). The fluorophore-conjugated secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 546 goat anti-rabbit IgG were obtained from Life Technologies (Carlsbad, CA, USA), the horseradish peroxidase-conjugated antibodies goat anti-mouse IgG and mouse anti-rabbit IgG were purchased from Rockland (Rockland, Limerick, PA, USA). HaCaT keratinocytes were kindly provided by R. Greinert (Buxtehude, Germany) and originate from P. Boukamp (Heidelberg, Germany).

2.2. Cell culture conditions and treatment

Human adult low calcium high temperature (HaCaT) keratinocytes were cultured in Dulbecco's modified Eagle medium (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Sigma, Steinheim, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were treated 24 h after seeding. Irradiation was done using a ^{137}Cs source (Gammacell 1000 Elite, Nordion, Ottawa, ON, Canada). Doxorubicin pulse treatment was performed for 2 h.

2.3. Immunocytochemistry

As surrogate markers for DNA double-strand break, formation of nuclear foci resulting from Ser139 phosphorylation of histone 2AX (γH2AX) and recruitment of 53BP1 was measured using immunofluorescence. To this end, cells were seeded onto cover slips, treated with the indicated doses and were fixed with 4% formaldehyde solution followed by an overnight incubation with methanol at −20 °C. Subsequently, cells were blocked in 5% BSA in 0.3% Triton X-100/PBS, incubated with primary antibodies (dilution 1:500, 16 h, 4 °C) and incubated with fluorophore-labeled secondary antibody for 90 min at RT in the absence of light. Cell nuclei were then counterstained with DAPI-containing Vectashield (Vector Laboratories, Burlingame, CA, USA) and analyzed with Olympus BX43 microscope (Olympus, Hamburg, Germany).

2.4. Alamar Blue Assay

Cell viability was determined using the Alamar Blue Assay. 48 h treatment, cells were incubated with 44 μM resazurin sodium salt (Sigma, Steinheim, Germany) in Dulbecco's modified Eagle medium w/o phenol red (Sigma, Steinheim, Germany) for 1.5 h. Fluorescence at 535 nm absorbance and 590 nm emission (Tecan infinite 200, Tecan, Männedorf, Switzerland), corresponding to the reduction of non-fluorescent resazurin to fluorescent resorufin, was analyzed to determine cell viability. Cell viability following genotoxin treatment is displayed relative to the respective untreated control, which was set to 100%. Changes in cell morphology were analyzed by light microscopy.

2.5. Impedance-based cell analysis using iCELLigence system

Cell analysis with the iCELLigence system (ACEA Biosciences, San Diego, CA, USA), which allows to record alterations in cellular impedance in real time, was performed according to the manufacturer's instructions. Impedance is the ratio of the voltage to the current in an alternating current (AC) circuit and corresponds to the resistance in a direct current (DC) circuit. Since cells function as insulators the number of cells is proportional to the measured impedance. Briefly, micro electrode-covered 8-well E-plates were filled with 200 μl complete medium per well to record background impedance. Then, 250 μl medium containing 5000 cells was added to each well. The appropriate cell