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Sensitivity of HaCat keratinocytes to diabetogenic toxins

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Abstract

Metabolic, genetic and environmental factors very likely play an important role in the development of skin lesions in diabetes mellitus. While these lesions are involved in secondary diabetes complications, various diabetogenic genotoxic agents may induce direct skin damage. In the present study we examined the potential of known diabetogenic agents (streptozotocin (STZ) and alloxan (AL)), with different mechanisms of action, for induction of direct injury in an immortal human keratinocyte HaCat cell line. In contrast to STZ, which induces alkylation of DNA, a genotoxic effect of AL is achieved through reactive oxygen species. We found that HaCat cells are highly sensitive to STZ, but not to AL. At a concentration of 10 mM STZ, cell viability decreased to $32 \pm 13\%$ of control ($P < 0.05$), as compared to $82 \pm 14\%$ with 10 mM of AL. Cells treated with 10 and 20 mM STZ showed a significant increase in apoptosis (3.9- and 6.7-fold), but not in necrosis, compared to naive cells ($P < 0.05$). In contrast to STZ, no increase in apoptotic and necrotic cell death was observed after AL treatment. Pretreatment with non-metabolizable 3-*O*-methyl glucose (3-OMG), which can blockade glucose transporter, or with poly(ADP-ribose) polymerase inhibitors (nicotinamide or 3-aminobenzamide), did not protect keratinocytes from STZ injury. Our results show that STZ, but not AL, is highly toxic to the HaCat cell line. Unlike insulin-producing cells, STZ-induced injury of immortal human keratinocyte HaCat cells is independent of the glucose transporters as well as of the activation of poly(ADP-ribose) polymerase.

Keywords: HaCat keratinocytes; Streptozotocin; Alloxan; Cytotoxicity; Apoptosis; Necrosis

1. Introduction

Different growth factor receptors, including insulin-like growth factor and epidermal growth factor, are predominantly expressed in basal keratinocytes in order to maintain tissue homeostasis [1]. In diabetic patients, changes in the levels of various growth factors such as insulin and insulin-like growth factors, combined with high blood glucose concentration, could induce tissue injury by disruption of the normal growth and differentiation pathways of the skin cells. It is generally accepted that the skin and connective tissue are involved in secondary diabetes complications, in the form of the development of distinct skin lesions and impaired wound healing. Moreover, hyperproliferative skin diseases, such as psoriasis, were reported to be associated with a tendency to develop diabetes [2], but the nature of these relationships is not fully understood.

On the other hand, different genotoxic diabetogenic agents can affect various cell types resulting in multiple disorders, including skin lesions. Keratinocytes are known to be sensitive to the direct cytotoxic effect of alkylating agents [3–5]. A potential common pathway of genotoxic drug action is believed to be caused by DNA strand breaks, and activation of poly(ADP-ribose) synthetase, leading to depletion of the NAD^+ pool in affected cells [6]. STZ is the most widely used agent for induction of diabetes in animals, causing powerful DNA alkylation [7]. Another well known diabetogenic agent is AL, which was the first cytotoxic compound reported to induce inhibition of glucose-dependent insulin secretion and pancreatic beta cell damage. In contrast to STZ, a genotoxic effect of AL seems to be achieved through reactive oxygen species, which

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Abbreviations: STZ, streptozotocin; AL, alloxan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 3-OMG, 3-*O*-methyl glucose; NA, nicotinamide; 3-ABA, 3-aminobenzamide; PARP, poly(ADP-ribose) polymerase; GLUT-2, glucose transporter-2; NP-40, nonidet P-40; FCS, fetal calf serum; FACS, fluorescent activated cell sorter.

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induce DNA fragmentation [8]. Although the cytotoxic effect of both of these compounds is known to be mostly beta cell specific, their actions could theoretically affect other cells, such as keratinocytes.

In the present study, we examined the possibility that STZ and AL, diabetogenic agents with different mechanisms of action, can directly induce keratinocyte injury. In order to eliminate the effects of various metabolic abnormalities associated with diabetes, all the experiments were conducted under controlled conditions using the immortal human keratinocyte HaCat cell line. This cell line was derived from normal human abdominal skin and expresses a relatively high level of cell differentiation [9]. Although HaCat cells display a transformed phenotype, they are non-tumorigenic and non-invasive. Moreover, HaCat cells have previously been used for reconstruction of a human skin equivalent [10]. On the other hand, HaCat cells may be considered as a model of hyperproliferative skin alterations, such as psoriasis, which is one of the most frequent skin lesions observed in diabetic patients [11].

2. Materials and methods

2.1. Materials

STZ, AL, hydrogen peroxide, 3-OMG, bovine serum albumin, nicotinamide (NA), 3-aminobenzamide (3-ABA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), Hoechst 33342, dimethylsulfoxide, trisodium citrate, nonidet P-40 (NP-40), trypsin, trypsin inhibitor, ribonuclease A and spermintetrahydrochloride were purchased from Sigma. Eagle's minimal essential medium, antibiotics, fetal calf serum (FCS), L-glutamine, Dulbecco's phosphate buffered saline, trypsin–EDTA solution and cell culture equipment were obtained from Biological Industries Ltd.

2.2. Cell culture

Experiments were performed with the immortalized human keratinocyte HaCat cell line which was previously described in detail [9]. The cells were propagated routinely in 75 cm^2 flasks using Eagle's minimal essential medium supplemented with 5% FCS and 1% antibiotics at 37° in 95% air and 5% CO_2. The culture medium was changed every 2–3 days. The cells (passages 25–32; subculture interval was 5–6 days) without mycoplasma contamination were used in all experiments.

2.3. Dose-dependent STZ, AL and H_2O_2 cytotoxicity

Viability of the HaCat cells after treatment with various doses of STZ was determined by measuring the activity of mitochondrial dehydrogenase enzymes of living cells in 96-well microtiter plates by MTT assay [12]. Briefly, equal numbers of cells (4×10^3) in 0.2 mL of medium were seeded in each well for 24 hr and then exposed for 1.5 hr to a glucose-free RPMI 1640 medium containing various concentrations of freshly prepared STZ solution (0–20 mmol/L), and AL (0–20 mmol/L). In order to estimate the HatCat reaction to oxidative stress, the cells (4×10^3 cells per well) were exposed to 0–400 μmol/L H_2O_2 for 2 hr in a CO_2 incubator. Following incubation with the three toxins, the cells were washed, and new culture medium was added for 72 hr. Twenty microliters of 5 mg/mL MTT were than added to each well for 3.5 hr. The medium was removed and formazan crystals were dissolved in 0.2 mL of DMSO. The amount of formazan was quantified by measuring optical density at 550 nm with an ELISA-reader 400 ATC (SLT Labinstrument).

2.4. Time-dependent cytotoxicity of STZ and AL

Briefly, 4×10^3 cells per well were exposed for 5, 10, 15, 20, 30, 60 and 90 min to a glucose-free RPMI 1640 medium containing 10 mM of STZ or AL. In a separate set of experiments, the cells were treated for 90 min with STZ degradation products which were prepared by incubating STZ in culture medium at 37° for 90 min. Following 72 hr of incubation, HaCat cell viability was estimated by MTT assay.

2.5. Effect of 3-OMG on STZ and AL cytotoxicity

The involvement of glucose transporters in cell sensitivity to STZ and AL was estimated by preincubation of HaCat cells in culture medium supplemented with 10 mM of non-metabolizable 3-OMG for 30 min prior to STZ or AL exposure. After preincubation, the cells were exposed for 90 min to a glucose-free RPMI 1640 medium containing various concentrations of STZ or AL (0–10 mmol/L). The effect of 3-OMG on STZ and AL cytotoxicity was determined by MTT assay.

2.6. Effect of PARP inhibitors 3-ABA and NA on STZ and AL cytotoxicity

HaCat cells were exposed to 10 mM of STZ or AL for 1.5 hr. Prior to the toxins treatment, the cells were incubated for 1 hr with 3 mM NA or 1 mM 3-ABA. The effect of poly(ADP-ribose) polymerase (PARP) inhibitors on cell survival following STZ or AL treatment was determined by MTT assay.

2.7. Estimation of apoptosis and necrosis by fluorescence microscopy

The numbers of apoptotic and necrotic cells were quantified using a fluorescent microscope, Axioscop-2 (Zeiss), as previously described [13]. Briefly, HaCat cells were cultured on glass slides for 48 hr to achieve 70%

confluence, and then exposed to 10 and 20 mM STZ or AL for 90 min. Following 48 hr of incubation, the cells adherent to the glass were stained with the DNA-binding dyes Hoechst 33342 (20 μg/mL) and PI (10 μg/mL) for 5 min at room temperature, covered with a coverslip, and examined with a fluorescence microscope with ultraviolet excitation at 340–380 nm. Viable cells were identified by intact nuclei and their blue fluorescence, necrotic cells by intact nuclei and red fluorescence, and cells in early or late phase of apoptosis by condensed fragmented nuclei and blue or red fluorescence, respectively. At least 1000 cells were counted for each condition. The frequency of apoptotic or necrotic cells was calculated as percentages of total cell populations. Cells treated with 5% ethanol served as a positive control for apoptosis.

2.8. Determination of apoptosis by flow cytometry

The 25×10^4 cells were seeded in 25 cm^2 tissue culture flasks and cultured for 24 hr. They were then exposed to 2.5, 5 and 10 mM STZ in glucose-free RPMI 1640 medium for 90 min and cultured for 48 hr. Flow cytometry analysis for DNA was performed on nuclei prepared from adherent and floating cells with a detergent-trypsin method and stained with PI [14]. Flow cytometry analysis of apoptosis was carried out in a fluorescence activated cell sorter FACScan (Becton Dickinson) using the Becton Dickinson Cell Quest program.

2.9. Statistical analysis

Analysis of variance (ANOVA) was utilized for evaluation of the statistical significance of differences between groups. The results are presented as mean values ± SD of independent, repeated experiments ($n = 3$–4). Experiments were done in triplicate, and $P < 0.05$ were considered significant.

3. Results

3.1. HaCat cells display different sensitivity to diabetogenic STZ and AL

Human keratinocyte HaCat cells displayed a progressive decrease of cell viability when cultured in the presence of different concentrations (2.5, 5.0, 10.0 and 20.0 mM) of freshly prepared STZ. At a STZ concentration of 5 mM, the cell viability estimated by MTT was slightly reduced, with a progressive 3-fold reduction at 10 mM and one of 8-fold at 20 mM. In contrast, HaCat cells were not affected by the presence of different concentrations of AL (2.5, 5.0 and 10.0 mM), and only exposure of the cells to high-dose AL (20 mM) induced a statistically significant decrease in cell viability, i.e. 63 ± 7.8% of control cells (Fig. 1). In addition, we found (Fig. 2) that HaCat cells were effectively protected against hydrogen peroxide, known to be produced in AL solutions [15].

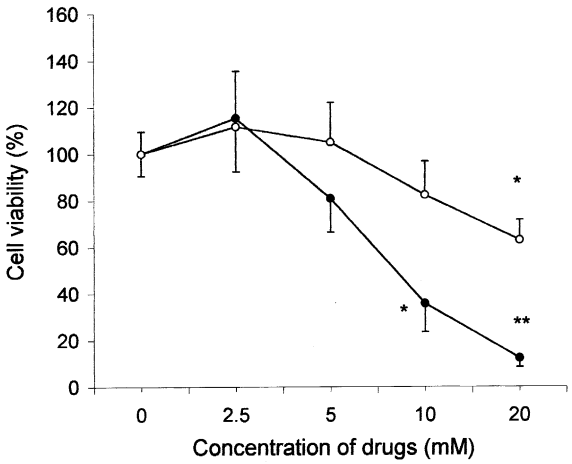

Fig. 1. Effect of STZ (black circle) and AL (open circle) on HaCat cell viability. Results are expressed as percent of viable cells compared to the control group (0 mM). Data are given as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group.

The time-dependent effect of 10 mM STZ on HaCat cells showed an influence on cell viability only after 60 min. In contrast to STZ, 10 mM AL had no statistically significant effect on HaCat cell viability (Fig. 3). However, when STZ was incubated in cell-free culture medium over 1.5 hr at 37° prior to cell treatment, no cytotoxicity was observed at toxin concentrations in a range of 2.5–20 mM (data not shown). Based on these data, all subsequent series of experiments were performed only with freshly prepared STZ solution.

3.2. PARP inhibitors 3-ABA and NA do not protect HaCat cells from STZ-induced injury

PARP activation which leads to NAD^+ depletion is known to be involved in pancreatic beta cell death induced by diabetogenic STZ [16]. Protection against beta cells

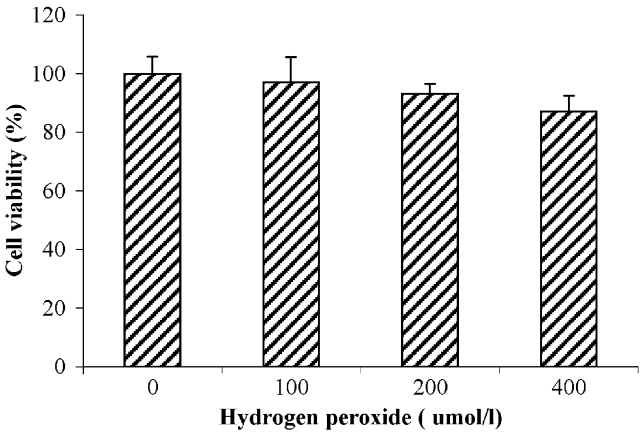

Fig. 2. Effect of hydrogen peroxide on HaCat cell viability. Results are expressed as percent of viable cells compared to the control group (0 μM). Data are given as mean ± SD of three independent experiments.

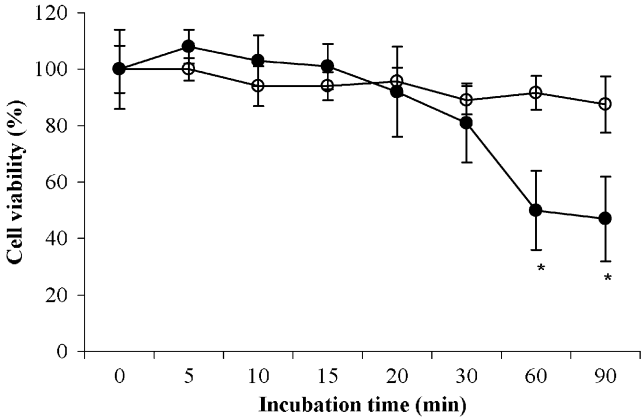

Fig. 3. Time-dependent effect of 10 mM STZ (black circle) and 10 mM AL (open circle) on HaCat cell viability. Results are expressed as percent of viable cells compared to the control group. Data are given as mean ± SD of three independent experiments. $^{*}P < 0.05$ compared to the control group.

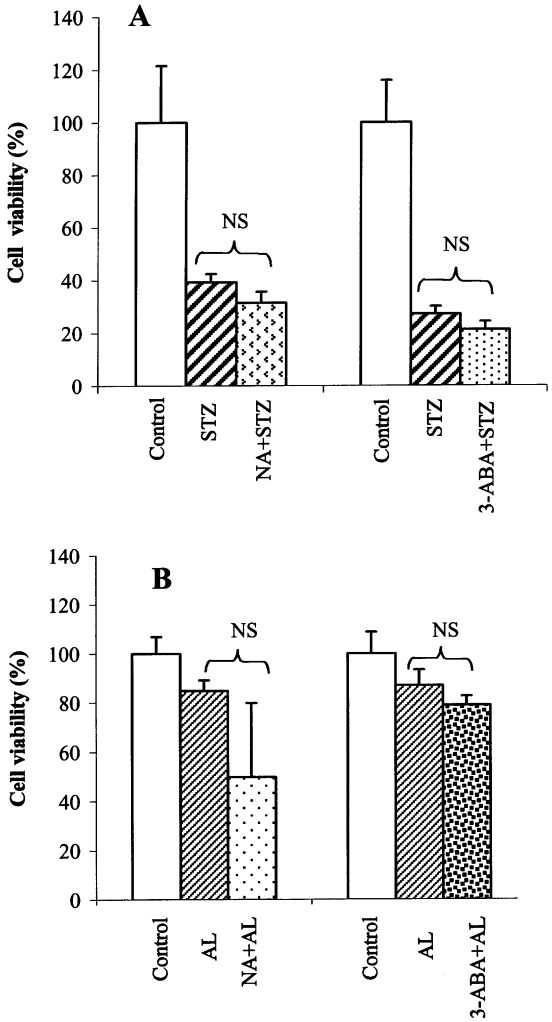

Fig. 4. Effect of NA and 3-ABA on HaCat cell sensitivity to STZ (A) and AL (B). Prior to STZ and AL treatment (10 mM), cells were incubated with 3 mM of NA or 1 mM of 3-ABA for 1 hr. Results are expressed as percent viable cells compared to the control group. Data are given as mean ± SD of three independent experiments. NS: non-significant differences between groups.

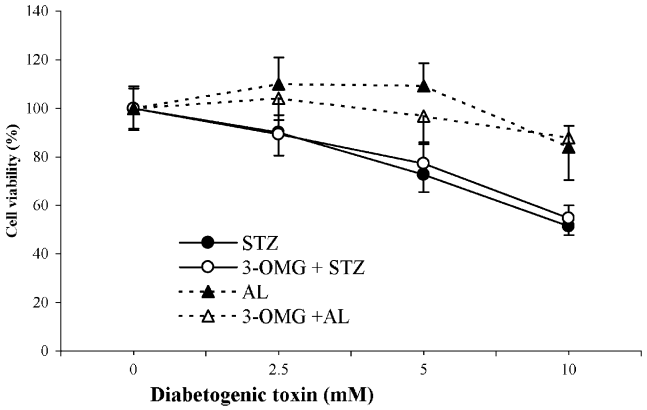

Fig. 5. Effect of 3-OMG on STZ and AL cytotoxicity in HaCat cells. Cells were exposed to 10 mM of 3-OMG for 30 min prior to 10 mM STZ or AL treatment. Results are expressed as percent of the viable cells compared to the control group. Data are given as mean ± SD of three independent experiments.

injury with STZ was induced by PARP inhibitors [17]. To elucidate the role of PARP in STZ-induced damage of HaCat keratinocytes, we used the PARP inhibitors 3-ABA and NA, at concentrations which were not cytotoxic for HaCat cells. We found that exposure of HaCat cells to 3 mM NA or 1 mM 3-ABA for 1 hr prior to treatment with 10 mM STZ or 10 mM AL had no protective effect against the diabetogenic drugs (Fig. 4).

3.3. 3-OMG has no effect on STZ and AL cytotoxicity in HaCat cells

Efficient killing of neuroendocrine cells by STZ, as well as by AL, was shown to be related to specific recognition of the drug as a transported substrate by glucose transporter-2 (GLUT-2) [8,18]. Since keratinocytes are known to express the various glucose transporters 1–5 [19], we speculated that there might be an involvement of glucose transporters in STZ-induced cytotoxicity. To estimate the possibility that the unmetabolizable 3-OMG blocks STZ transport into keratinocytes via glucose transporters, HaCat cells were

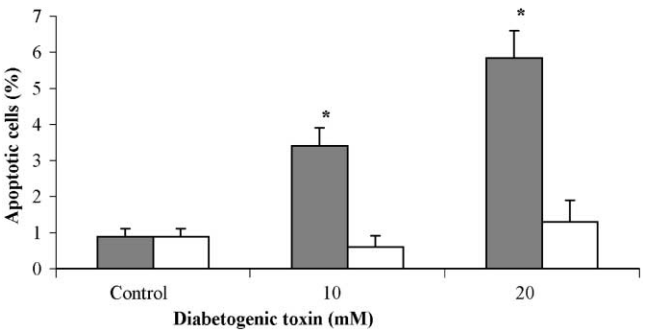

Fig. 6. Effect of STZ (black bar) and AL (open bar) on apoptosis in HaCat cells (microscopic analysis). Results are expressed as percent of apoptotic cells estimated by fluorescence microscopic assay. Data are given as mean ± SD of four independent experiments. $^{*}P < 0.05$ compared to the control group.

incubated with 10 mM 3-OMG for 30 min prior to STZ or AL exposure. In contrast to pancreatic beta cells, [18,20], 3-OMG did not have a significant protective effect on the HaCat cells (Fig. 5).

3.4. STZ induces apoptosis but not necrosis of HaCat cells

STZ is known to cause pancreatic beta cells death through apoptosis and necrosis [17]. The major differences between these two types of cell death is that apoptosis is a genetically and metabolically controlled cell death, while necrosis provokes cell death through acute injury. To explore the characteristics of STZ-induced cell damage in human keratinocytes, the frequency of apoptotic and necrotic cells was studied by fluorescent microscopy of HaCat cells stained with Hoechst 33342 and PI. The level of spontaneous necrosis and apoptosis in HaCat cells was found to be 0.3 ± 0.1 and $0.89 \pm 0.22\%$, respectively. When the cells were cultured in the presence of different

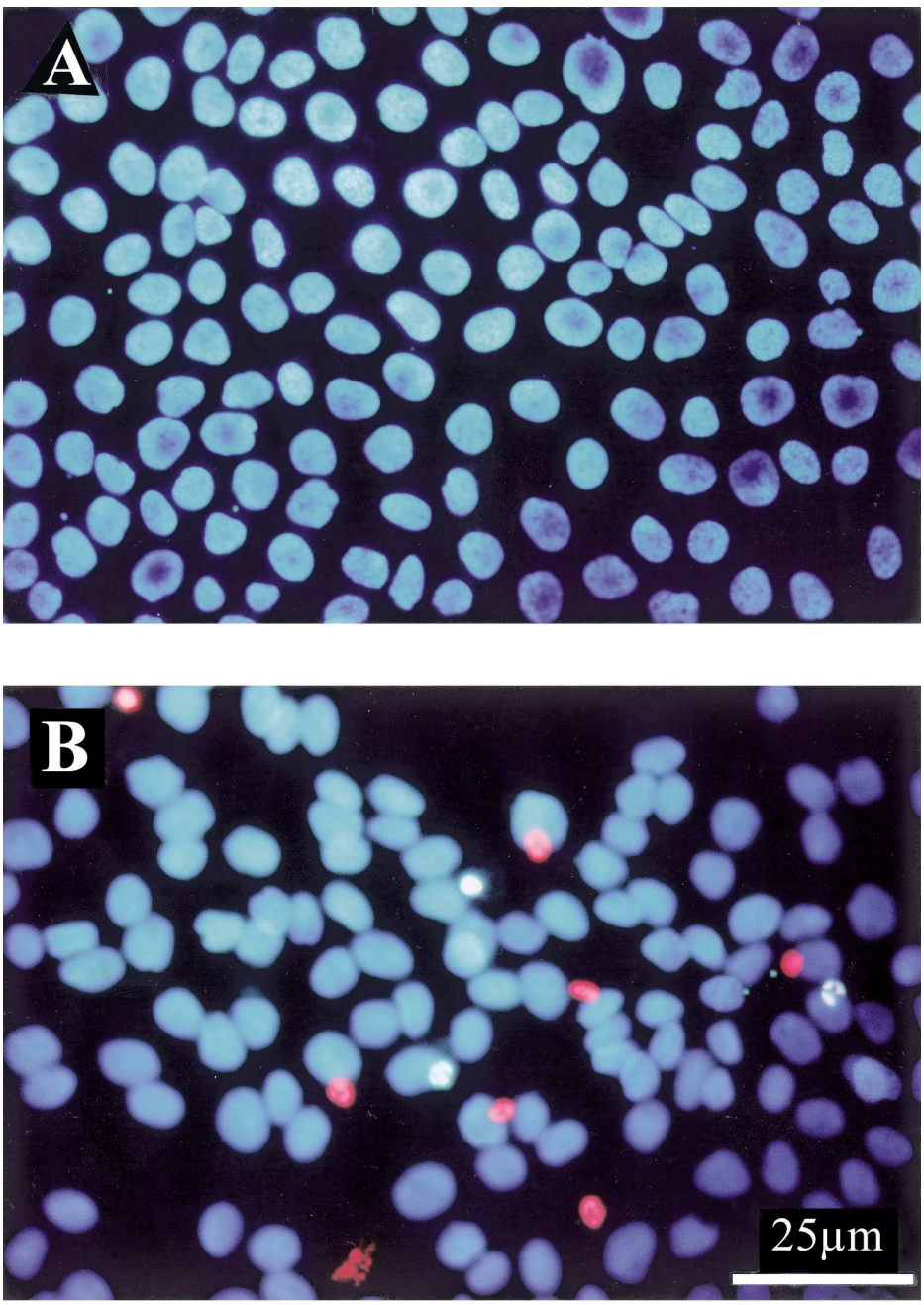

Fig. 7. Fluorescence microscopy of HaCat keratinocytes stained with Hoechst 33342 and PI before and after STZ treatment. (A) Hoechst 33342-positive (blue) intact nuclei of untreated HaCat cells; (B) Hoechst-33342-positive (blue) condensed and fragmented nuclei (early apoptosis), PI-positive (red) condensed and fragmented nuclei (late apoptosis) of HaCat cells after treatment with 20 mM STZ for 90 min. The figures is representative of four separate experiments.

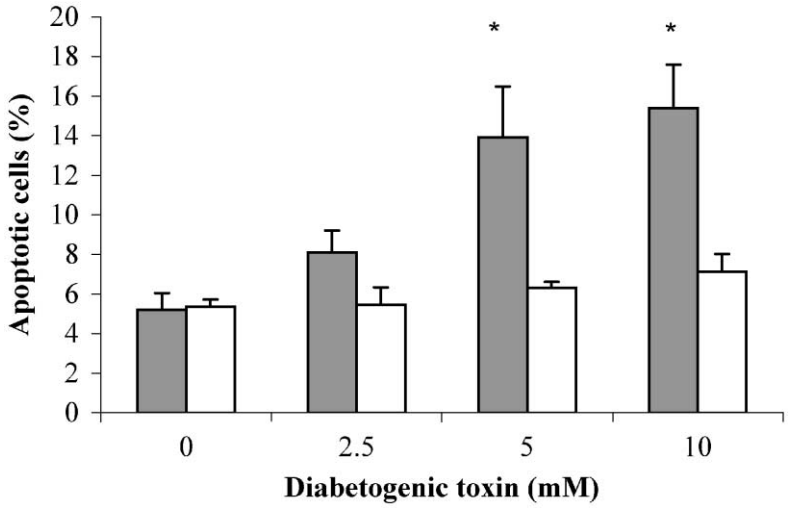

Fig. 8. Effect of STZ (black bar) and AL (open bar) on the level of apoptosis in HaCat (FACS analysis). Results are expressed as percent of apoptotic cells estimated by PI staining and FACS analysis. Data are given as mean ± SD of three independent experiments. $^{*}P < 0.05$ compared to the control group.

concentrations of STZ or AL, no statistically significant effect on necrosis was observed. However, the frequency of apoptotic cells was increased from 3.9- to 6.7-fold following treatment with 10 and 20 mM STZ, respectively; and no statistically significant increase in apoptotic cell death was observed after treatment with AL (Fig. 6). Fig. 7 presents the HaCat cells at early and late stages of apoptosis, indicating that the rate of apoptosis induced by STZ is not synchronized. Fluorescent activated cell sorter (FACS) analysis of apoptosis was performed in addition to the microscopic study. We found that increasing concentrations of STZ resulted in dose-dependent activation of apoptotic cell death from 1.6- to 3-fold at 2.5 and 10 mM STZ compared to untreated HaCat cells ($5.2 \pm 0.85\%$). In contrast, AL induced only very small activation of apoptotic death (Fig. 8). The discrepancy between the level of apoptosis seen by flow cytometry in untreated cells, and that observed by microscopy, probably results from estimating both adherent and floating cells by FACS, and only adherent cells by microscopy.

4. Discussion

Our study shows that HaCat cells are significantly more sensitive to the cytotoxic effect of STZ compared to that of AL. These findings might result from the existence of different pathways by which these two drugs exert their deleterious action: while AL induces DNA damage primarily via reactive oxygen species [8], STZ causes DNA strand breaks by alkylation [21]. Indeed, previous reports described the high sensitivity of keratinocytes to alkylating agents [3,4]. On the other hand, the lower sensitivity of HaCat cells to AL probably depends upon effective defense mechanisms of the keratinocytes against reactive oxygen species. Such property of the skin barrier is crucial for conservation of the body structural integrity. Here we have shown that HaCat keratinocytes display significant defense properties against hydrogen peroxide, a compound produced by AL [15]. The low sensitivity of cultured human keratinocytes to peroxide-induced (e.g. H_2O_2) oxidative stress, has been previously reported by others [22]. In contrast to keratinocytes, insulinoma cells, are highly sensitive to hydrogen peroxide, probably due to their poor antioxidative defense systems [23]. The strong antioxidant potential and the high enzymatic activity acting directly and indirectly on reactive oxygen species in keratinocytes [24], may very likely prevent the AL-induced cell damage. This is consistent with evidence that administration of superoxide dismutase and catalase effectively protects against AL-induced islet cell DNA breaks. However, STZ-induced islet DNA strand breaks were unaffected by radical scavengers, such as those of superoxide dismutase and catalase [16]. Another possible mechanism which may explain the decreased sensitivity of HaCat to AL, could be a lower level of NAD(P)H, a cell compound shown to act as a non-enzymatic activator of AL [25].

The pancreatic beta cell sensitivity to STZ cytotoxicity is known to depend on the level of GLUT-2 expression, which is a major type of glucose transporter in pancreatic islet cells. Such dependency is associated with the specific recognition of STZ as a GLUT-2 transportable substrate [18,20]. In order to evaluate the role of glucose transporters in HaCat cells, we competitively inhibited its binding to STZ by the addition of 3-OMG, a molecule which binds to, and is transported by glucose transporters, but is not metabolized by beta cells. Unlike insulin-producing cells, the lack of a protective effect of 3-OMG on STZ cytotoxicity suggests the existence of a glucose transporter-independent pathway for STZ-induced damage of HaCat keratinocytes. Our data are in agreement with recent

reports suggesting that STZ may be toxic for glucagonoma cells, acting through a pathway independent of the glucose transporters [26]. It should also be noted that STZ rapidly decomposes at physiological pH, and its degradation products, in the form of carbenium ions, act as powerful alkylating agents [7,16,27]. Thus, it is possible that the intracellular penetration of these ions—rather than the intact STZ molecules—is responsible for the drug's cytotoxicity in HaCat cells. Our hypothesis was confirmed by experiments showing that HaCat cells are not affected by a relatively short incubation (i.e. 30 min) with a high concentration of STZ, while a substantial reduction in cell viability was found following a longer incubation period (1 hr). It would appear that at least 60 min of incubation is necessary to produce cytotoxic levels of carbenium ions during drug decomposition, since the half-life of STZ in solution at neutral pH is approximately 1 hr [28,29]. However, incubation of STZ for 90 min prior to HaCat cell treatment completely reduced its cytotoxicity, suggesting a relatively short half-life of STZs degradation products. These data are supported by the previously reported findings, which showed that the degradation products obtained after incubation of STZ for 24 hr at neutral pH are not diabetogenic to rats [30].

It is generally believed that alkylating agents cause cell death by the induction of DNA strand breaks, followed by PARP activation. Over-activation of this enzyme causes depletion of the intracellular NAD^+ pool, eventually resulting in cell death [6]. However, the role of NAD^+ pool in cell death is not completely understood as administration of NA, a precursor of NAD and an inhibitor of PARP, to rats concurrently with STZ, protected the beta cells, as reflected by the unaffected proinsulin synthesis, but induced development of islet cell tumors [31]. Moreover, several observations showed that depletion of NAD^+ in rat and human keratinocytes exposed to alkylating agents is not a prerequisite for cell death [4,32], suggesting a major role for an NAD^+-independent pathway of keratinocyte death by alkylating agents. In order to evaluate the role of PARP activation in STZ-induced damage, we incubated HaCat keratinocytes before STZ treatment with inhibitors of PARP (NA or 3-ABA). This procedure did not affect cell viability, indicating that PARP activation is not a major cause of STZ-induced cytotoxicity in HaCat keratinocytes. Moreover, our data show that STZ induces activation of apoptotic, but not necrotic, cell death, and that PARP inhibitors fail to protect HaCat cells from apoptosis. This is consistent with the observation that NA protects human pancreatic beta cells from necrosis, but not against apoptosis induced by toxic agents [17]. In summary, our results suggest that diabetogenic agents can directly induce cell injury in skin cells. STZ, but not AL, is highly toxic to human keratinocyte HaCat cells; however, unlike the insulin-producing cells, the STZ-induced injury of keratinocytes is independent of the glucose transporters and of activation of PARP.

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