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In vitro toxicity studies with mitomycins and bleomycin on endothelial cells

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Pulmonary side effects are increasingly observed as dose-limiting toxicity (DLT) of cancer treatment. The available preclinical models have a limited predictive value for lung toxicity in humans. We have attempted to elucidate potential mechanisms involved in these reactions, by studying the effects on cells, possibly involved in these reactions after *in vitro* exposure to drugs with known lung toxic effects. We have investigated the effects of bleomycin (BLM), mitomycin C (MMC), KW-2149 and its two known metabolites, M16 and M18, on oxygen radical production by granulocytes, on cytokine production: interleukin (IL)-6, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α by a human macrophage cell line (THP-1), by human endothelial cells (HVEC and HMEC) and a human colorectal cancer cell line (DLD-1), and on the cytotoxicity on endothelial cells in both confluent and non-confluent culture. The generation of oxygen radicals by normal and pre-stimulated granulocytes was not increased after preincubation with any of the drugs, at the concentrations tested. None of the cytokines (IL-6, TNF-α or TGF-β) was found significantly increased in culture medium after exposure to any of the mitomycins. This was in contrast with the effect of BLM incubation, causing a rise in TGF-β concentration. Both types of endothelial cells showed a dose-dependent, exposure duration-dependent, proliferation inhibition for all agents tested. This inhibitory effect was clearly proliferation dependent as shown by the increased inhibition in semi-confluent as opposed to confluent endothelial cell cultures. Both mitomycins tested were more cytotoxic than BLM to both confluent and proliferating endothelial cells.

Key words: Angiogenesis, bleomycin, endothelial cells, KW-2149, mitomycin C, pulmonary toxicity.

Introduction

The development of pulmonary toxicity by cytotoxic agents is an increasingly important problem.[1] This toxicity is best known for bleomycin (BLM).[2–4] Other agents with pulmonary side effects are mitomycin C (MMC)[5–10] and the nitrosoureas.[11] With the alkylating agents, lung toxicity is a rare event, often related to their use in high-dose regimens.[12,13] High-dose chemotherapy regimens against solid tumors have led to new types of toxicity, and have made toxic effects both more frequent and more severe.[14–16] The incidence of veno-occlusive disease, both in the liver and the lung, and the increased interstitial type of pulmonary damage are examples of these reactions. The position of the vasculature predisposes it to injury after exposure to a high concentration of circulating drugs.[17] A possible 'vascular injury' mechanism as part of the pathophysiology of drug-induced lung toxicity is gaining acceptance. The pulmonary endothelium is the first observable site of injury, preceding further inflammation and fibrosis following the administration of BLM.[18] MMC can cause a delayed-onset, progressive pulmonary vascular injury resulting in veno-occlusive disease and pulmonary hypertension.[19,20] We described the development of interstitial-type pulmonary damage as the main toxic event during a phase I trial with an MMC analog, KW-2149.[21–24]

Others have observed similar effects with another MMC analog, BMY 25067.[25,26] The role attributed to the lung in both the accumulation and the metabolism of xenobiotics is undergoing important changes.[27,28] In man, the pulmonary endothelial surface adds up to some 70 m^2 and this assures interaction with xenobiotics present in the blood. The lung exerts a first-pass effect for compounds injected i.v. and influences the kinetics of the drug availability on the arterial side. With regard to cytotoxic drug-induced lung toxicity, BLM has been studied most intensively and ultimately has been considered as a paradigm for drug-induced pulmonary damage.[29–39] What has been learned from these experiments? First, endothelial cell damage is the earliest morphological event. Second, the pathology

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and biochemistry of these events is comparable after i.v., s.c. or i.t. administration, the time course and concentration dependency being different. Third, dose-dependency experiments have shown endothelial cell dysfunction after both high- and low-dose exposure, but fibrosis only after high-dose exposure. Fourth, cytotoxic agents, not associated lung toxicity, cause predictable lung toxicity after i.t., and not so after s.c. or i.v. administration. Fifth, protracted infusion improves lung tolerance both in models and in humans. Sixth, oxygen radical formation is part of the pathophysiology of lung injury.

We have studied the effects of four mitomycins and BLM on granulocytes, macrophages and endothelial cells, with the intention to elucidate underlying mechanisms of these toxic reactions and to suggest preclinical models. The effects of chemotherapy on endothelial cells have recently been studied because of the interest in the process of tumor angiogenesis.[40-42] Angiogenesis is important because it is considered a prerequisite for tumor growth. Newly developed vessels supply the tumor with oxygen, nutrients and growth factors, enabling growth, invasion and the development of metastases. These tumor vessels may also limit the penetration of drugs. This is due to the erratic anatomy of these vessels, the increased interstitial pressure and the limited strength of the vessel wall. Tumor-associated endothelial cells differ from those in normal vessels. The proliferative activity in tumor-associated endothelial cells is about 50-fold greater than their normal counterparts. Considering the fact that proliferating cells are more susceptible to cytotoxic agents, it might be that chemotherapeutic drugs influence tumor angiogenesis by interfering with the proliferating endothelial cells.[43-48] This hypothesis considers the dose-intense exposure of the tumor-associated endothelial cells, whereas the tumor cells are exposed to much lower concentrations under hypoxic conditions. The direct effects of cytotoxic agents on endothelial cells have remained largely uninvestigated. We have studied the cytotoxicity of mitomycins and BLM in confluent and near-confluent endothelial cultures.

Material and methods

Cells

Granulocytes. Granulocytes were isolated out of peripheral blood collected in EDTA tubes. After centrifugation, the buffy coat was collected, washed in Hank's balanced salt solution without calcium or magnesium ($HBSS^-$), and layered on a discontinuous Percoll gradient with densities of 1.077 and 1.088 g/ml. The granulocyte layer was collected and washed. Purity was always greater than 87% and viability, tested with the Trypan blue exclusion test, was in excess of 90%. Peripheral blood was collected from human volunteers (group A, $n = 6$) and two different patient groups.

The first patient group (group B, $n = 5$) was being treated with BLM-containing chemotherapy but without any evidence of pulmonary toxicity. The second group of patients (group C, $n = 4$) consisted of patients receiving BLM-containing chemotherapy but with some degree of pulmonary damage (above 20% of normal CO diffusion capacity).

Endothelial cells. The human microvascular endothelial cell line (HMEC) was cultured in MCDB 131 medium supplemented with 10 ng/ml EGF (Collaborative Biomedical Products, New York, NY; 40001), 1 μg/ml hydrocortisone (Sigma, St Louis, MO; H0888) and 20% fetal calf serum (FCS). Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical vein as described by Jaffe *et al.*[50] They were maintained in M199 medium supplemented with 20% FCS, ECGS 0.01 mg/ml (Sigma), heparin 1 U/ml, penicillin 100 U/ml and streptomycin 100 g/ml. Second and third passage cells were used. All endothelial cells were maintained in a humidified 5% CO_2 incubator at 37°C. Subcultures were obtained by the use of 0.05% trypsin–0.2% EDTA.

Tumor cells. As a control for BLM resistance, the DLD-1 cell line, a human colorectal adenocarcinoma cell line (ATCC, Rockville, MD), was used. This tumor cell line was cultured in DMEM medium supplemented with glutamine stock solution and 10% heat-inactivated FCS. Cells were cultured in a 5% CO_2 incubator at 37°C. The doubling time for the DLD-1 cells was between 24 and 30 h. For subculture, the cells were treated with 0.05% trypsin–EDTA for 2 min at 37°C to obtain a cell suspension.

Macrophages. Since it is difficult to obtain large amounts of human pure monocytes, we used the THP-1 cell line (ATCC) as macrophage cell line.[51,52] Cells were cultured in DMEM supplemented with 5% FCS and cultured in a 5% CO_2 incubator at 37°C. Cells were seeded at a density of 5×10^5 ml.

Drugs

BLM (molecular weight 1500) (Roger Bellon, Paris, France) consists mainly of a mixture of BLM A_2 and

BLM B_2. BLM was dissolved in 5 ml saline and further diluted in a 0.9% NaCl solution to a final concentration of 100 mg/ml. A new solution was made prior to each set of experiments. MMC (molecular weight 334) (Kyowa Hakko, Tokyo, Japan) was dissolved with normal saline and diluted up to a concentration of 10 mg/ml.

KW-2149 (MW 598), M16 and M18 were a kind gift from Kyowa Hakko. These were dissolved up to concentrations of 10 mg/ml. For the cytotoxicity experiments each stock solution was further diluted with the appropriate medium up to concentrations ranging from 120 to 0.012 μg/ml. In order to achieve conditions comparable to the *in vivo* situation the range of concentrations tested reflected the ones obtained in patients.

Cytotoxicity assay

All cells were cultured in 5 cm^2 Falcon dishes. Before each test, the cell culture was split and cells were distributed into 96-well plates. The cytotoxicity testing for endothelial cells was performed with both confluent and semiconfluent cultures. In order to obtain confluence two parameters were changed. First, the seeding density was increased for confluent cultures; 20 000 versus 10 000 cells/well for the semiconfluent condition. Second, even with an increased seeding density confluence was reached only after a more prolonged culture time, day 4–7, before the cytotoxic test could be performed. Cytotoxicity tests on semiconfluent cultures of HMEC were performed on the day after distribution into the wells. For HUVEC semiconfluency was reached after 2–3 days. The DLD-1 cells were used for cytotoxic tests on day 2. The cell survival was determined using the Alamar Blue assay (Alamar Biosciences, Sacramento, CA).[53] Alamar Blue is a water soluble, non-toxic dye for living cells and a fluorimetric indicator. The fluorescence of this dye changes after chemical reduction of the medium resulting from cell proliferation. The intensity of the fluorescence is proportional to the cell number. In initial experiments this was shown to be true for endothelial cells as well. When distributed into the wells, the cells were grown to subconfluency or confluence and the percentage of confluence was always assessed with an inverted microscope. The wells with 60–70% of confluence were considered as semiconfluent cultures. Thereafter cells were incubated for 2 h with different concentrations of drugs. Semiconfluent HMEC had an additional exposure time of 18 h. Each concentration was tested at least in triplicate and every experiment was repeated minimally three times. After 2 h incubation with the drug, the medium was completely replaced and cells were cultured with normal medium for 16 h to reveal a cytotoxic effect. Cell survival was measured colorometrically with the Alamar Blue technique. The normal medium was removed and replaced with medium containing 2.5% of Alamar Blue. The plates were incubated at 37°C and after 6 h the intensity of fluorescence was measured with a Cytofluor™ 2300 Fluorescence Measurement System.

Superoxide radical production

Generation of superoxide anion radicals by granulocytes was measured as superoxide dismutase-inhibitable reduction of ferricytochrome C as described.[54]

Briefly, 0.1 ml 10^7 granulocytes were activated with 0.1 ml 10^{-8} M or 10^{-10} M phorbol myristate acetate (PMA; Sigma) in 1 ml $HBSS^+$ at 37°C, in the presence of 0.4 ml ferricytochrome C (2.5 mg/ml; Boehringer Mannheim, Mannheim, Germany) with 0.4 ml superoxide dismutase (300 mg/ml; Sigma) as blank in a total volume of 1.0 ml. The superoxide generation was stopped after 30 min by adding 1 ml ice cold water $HBSS^-$. Reduced ferricytochrome C was measured spectrophotometrically at 550 nm. Results were expressed as nanomoles of superoxide anion released by 10^6 PMN/ml.

In vitro cytokine production

The production of cytokines, putatively involved in the process of lung fibrosis, was measured in the supernatants of different cells before and after incubation with different drugs at different concentrations. These experiments were performed with the TPH-1, HUVEC, HMEC and DLD-1 cells. Cells were distributed into 6-well plates at a seeding density of 200 000 well. They were then incubated with 0.12, 1.2 and 120 mg/ml of BLM, MMC and KW-2149. Exposure time was set at 12, 24 and 48 h for each of the three drug concentrations. Each incubation-time combination was performed in triplicate. Cytokine production was determined in the supernatants for interleukin (IL)-6, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β. The supernatant was isolated, filtered and stored immediately at -80°C until analysis. All cytokines were quantified with an ELISA technique. These used monoclonal anti-human antibodies for IL-6 and TNF-α, and a polyclonal anti-human antibody for TGF-β.

Statistical analysis

The concentration effect of different drugs on endothelial cell cultures was analyzed by comparing the fluorescence of control and treated cells. The significance of this difference was calculated with a paired *t*-test. To assess the cytotoxic effect on endothelial cells in a 5-fold concentration range, the IC_{20} and IC_{50} values were determined from the dose-response curves. Mann-Whitney *U*-testing was performed to compare the individual measurement points, comparing the dose-response curves of semiconfluent and confluent cultures. Calculations were performed by the Statview™ version 4.0 package for the Macintosh™.

Results

Superoxide production

Superoxide production was measured after 30 min incubation of both unstimulated and PMA pre-stimulated granulocytes (PMA 10^{-8} and 10^{-10} M), with three different concentrations for each drug. The results are expressed as nanomoles of superoxide anion released per 10^6 PMN/ml. The superoxide production by granulocytes from healthy volunteers is shown in Table 1. Each drug and each concentration was tested with granulocytes from three different volunteers. There was no influence on the production of superoxide radicals, either in the stimulated or in the unstimulated condition, by any of the five drugs at any of the tested concentrations.

Identical experiments were performed with granulocytes originating from five patients receiving BLM-containing chemotherapy and without evidence of lung toxicity with a total dose of BLM administered above 150 mg and with granulocytes from four patients treated with a comparable dose of BLM but with evidence of pulmonary toxicity. Again no difference in superoxide production was observed, neither under baseline conditions nor after PMA stimulation (data not shown) in group B patients. A rise in superoxide production was observed in one of the patients of group C (with lung toxicity). This observation was observed on two separate samples of this patient, one during chemotherapy and the second after chemotherapy.

Endothelial morphology

Morphological effects of MMC exposure became visible after 24 h. These effects were concentration dependent and most pronounced in the semiconfluent cultures. In the semiconfluent endothelial cell cultures these changes appeared by 24 h at the concentration of 1.2 μg/ml for KW-2149, 12 μg/ml for MMC and 120 μg/ml for BLM.

Cells detached by 24 h and this increased to a maximum after 5 days. The monolayers became less cellular and at the highest concentration of KW-2149 (120 mg/ml) virtually no adherent cells remained by day 5. Confluent cultures were clearly less sensitive—even at 120 μg/ml for BLM only minor alterations were observed, but apparent injury was observed at 12 mg/ml for MMC and KW-2149. This became apparent by 24 h by the loss of the cobblestone appearance and the irregular cell borders. By day 5 these effects became more pronounced.

Endothelial proliferation

Concentration effect (Figure 1 and Table 2). The significance in the difference between the survival of control and treated cells was calculated using a paired *t*-test and is presented in Figure 1. The endothelial cell

Table 1. Unstimulated and pre-stimulated superoxide anion production in human PMN from healthy donors after incubation with antitumor agents

Concentration	Unstimulated	PMA 10^{-8}	PMA 10^{-10}
BLM			
0	8.5	47.9	11.6
0.1	0	36.2	4.6
1.0	8.0	54.7	10.2
10	5.2	55.5	8.5
KW-2149			
0	4.1	40.2	8.0
0.1	4.2	45	4.1
1.0	3.3	44.5	8.3
10	5.6	45.1	7.2
MMC			
0	4.1	40.2	8.0
0.1	4.5	46.3	4.9
1.0	5.6	42.8	6.9
10	4.6	44.7	6.9
M-16			
0	4.1	40.2	8.0
0.1	6.1	49.2	5.6
1.0	5.5	42.3	6.6
10	5.2	44.7	7.0
M-18			
0	4.1	40.2	8.0
0.1	4.8	45.2	6.3
1.0	4.4	44.8	7.1
10	3.2	44.2	8.2

Concentrations are expressed as mg/ml. Results are shown as the mean from three measurements.

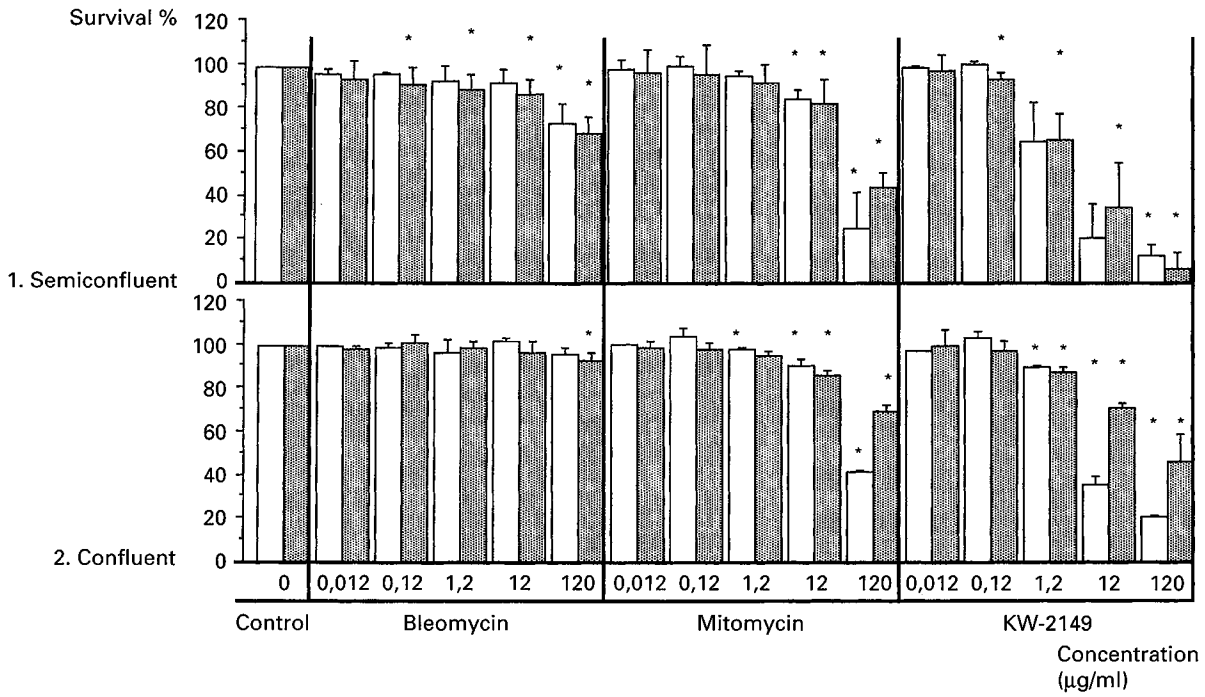

Figure 1. The influence of concentration of the cytotoxic antibiotics on confluent or semiconfluent endothelial cells. Significant difference between survival of control and treated cells: $*p<0.05$. HUVEC, open bars; HMEC, gray bars.

Table 2. The cytotoxic effect of BLM, MMC and KW-2149 for the different endothelial cells and DLD-1

	HMEC						HUVEC				DLD-1	
	Semi/2 h		Semi/18 h		Confluent		Semi/2 h		Confluent			
	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}
BLM	59	–	7	106	–	–	84	–	–	–	–	–
MMC	20	106	4	44	52.5	–	23	77	37	103	17	112
KW-2149	0.05	7.5	0.02	1.0	7.5	104	1.2	6	4	9	1.2	12

survival decreases proportionally with increasing drug concentration. The confluent cultures of HMEC and HUVEC are resistant to BLM. The difference in EC survival for confluent versus semiconfluent cultures is only borderline significant for BLM. KW-2149 caused an apparent cytotoxic effect on both confluent and semiconfluent HMEC and HUVEC cultures at concentrations much lower than for any of the other drugs.

In Table 2 the concentrations of the different drugs required to reduce the fluorescent intensity of the Alamar Blue signal by 20 and 50% are shown.

The 50% reduction of Alamar Blue signal for KW-2149 occurred at a concentration observed in patients. Within this concentration range MMC was observed to exert a cytotoxic effect resulting in a 20% decrease in survival. The IC_{50} for BLM was not attained within the concentration range investigated. A 20% reduction in survival for semiconfluent cultures occurred at 59 μg/ml.

Analysis of cytotoxicity in confluent and semiconfluent endothelial cultures (Figure 2). In general the sensitivity of endothelial cells for all three antitumor agents, within concentration range studied, was more pronounced in the semiconfluent than in the confluent cultures. The differences between the dose-response curves for the confluent and the semiconfluent cultures as well as sensitivity of tumor cell line DLD-1 is presented in Table 2 and Figure 2. The dose-dependent antiproliferative effect was observed for all cultures but the p values calculated for corresponding doses are more significant for the semiconfluent cultures. The IC_{20} and IC_{50} values for two models of culture also differ significantly. The dose-response

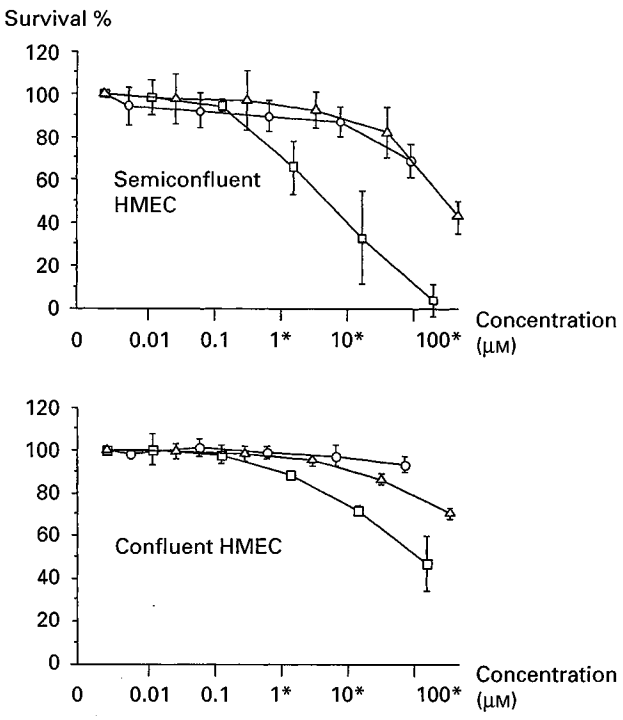

Figure 2. Comparison of the cytotoxicity of BLM, MMC and KW-2149 for HMEC (2 h of incubation). *Significant difference between toxicity of BLM, MMC and/or KW-2149 at concentrations marked. (○) BLM, (□) KW-2149 and (△) MMC.

curves shown for HMEC demonstrate a significant difference with a p value less than 0.05. The confluent endothelial cell cultures are not affected by exposure to BLM even at the highest concentration studied. The survival curves of both endothelial cells and DLD-1 cells cultured with BLM are not significantly different. The non-proliferating endothelial cultures are, however, more sensitive to other drugs tested. The most toxic agent for the confluent endothelial cells is KW-2149 with IC_{20} and IC_{50} values of 7.5 and 104 μg/ml for HMEC. The toxic effect exerted by MMC is also prominent but less so than for KW-2149 (IC_{20} = 52 μg/ml).

The sensitivity of DLD-1 cells for MMC and KW-2149 seems to be comparable with sensitivity of endothelial cell cultures towards these drugs. Interestingly, KW-2149 and BLM are even more toxic to proliferating endothelial cells than to proliferating tumor cells.

Effect of time of incubation on cell survival (Table 2). To investigate the influence of exposure time on endothelial cell proliferation, the HMEC cultures were incubated with each drug for 18 h instead of the standard 2 h exposure used in the other experiments. The cytotoxic effect exerted on semiconfluent cultures at 18 h of incubation starts to occur at much lower concentrations than after a 2 h exposure. The IC_{20} and IC_{50} values for 18 h incubation with drugs are presented in Table 2.

Comparative analysis of endothelial toxicity of BLM, MMC and KW-2149 (Figures 2 and 3). To compare the endothelial toxicity of the three drugs, Figure 3 shows cell survival after different concentrations. The concentration for each agent is expressed in μM concentrations, levels which did not coincide precisely for each agent. Therefore the comparison of the cytotoxic effect of drugs had to be performed within an extrapolated concentration range. BLM has the highest molar mass (1399), next to KW-2149 (598) and MMC (334). Within the concentration range studied, KW-2149 was observed to be the most potent toxic agent in endothelial cell cultures. Its toxic effect exerted on semiconfluent cultures in the highest concentration range is significantly higher than that caused by BLM (p = 0.0027) and MMC (p = 0.0027).

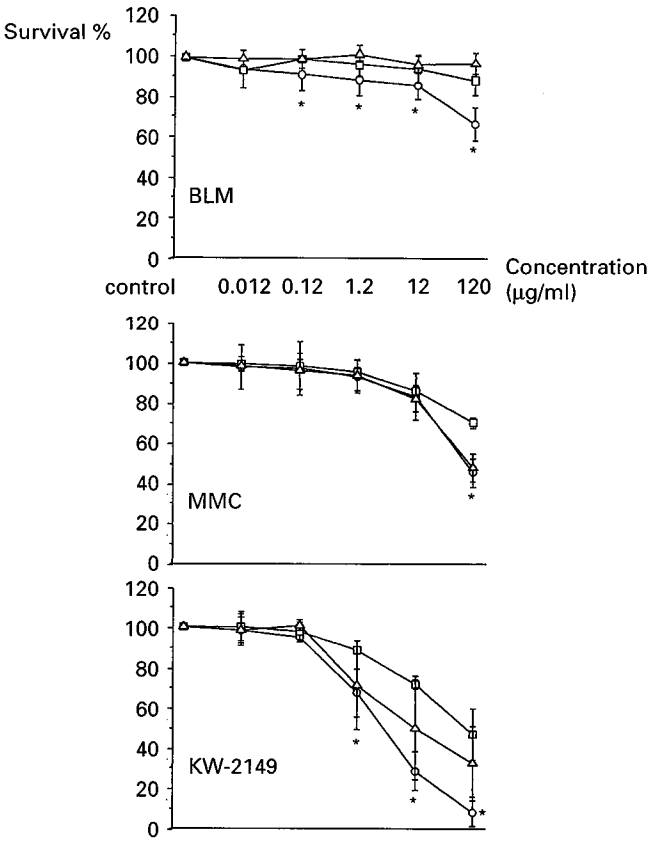

Figure 3. Comparison of the cytotoxicity of BLM, MMC and KW-2149 on confluent and semiconfluent HMEC and DLD-1 (*p<0.05). (○) HMEC semiconfluent, (□) HMEC confluent and (△) DLD-1.

The cytotoxic potential exerted by KW-2149 on confluent cultures is also significantly higher than for other drugs ($p < 0.05$). The toxicity of BLM and MMC towards semiconfluent cells is comparable in the concentration range which was tested, whereas MMC seems to be more toxic to confluent, non-proliferating cultures ($p = 0.039$).

Discussion

The importance of pulmonary complications as a dose-limiting toxicity of cancer treatment has been recognized. Recognition of lung toxicity related to the use of BLM and MMC, as well as experimental evidence of endothelial injury underlying these pulmonary changes, has led to these toxicity studies.[32,34,35,39,40,55-59] The damage caused by antitumor agents against normal host cells, on the other hand, might not only explain the toxicity profile of a particular agent, it might also be responsible for part of its *in vivo* efficacy.[46,47] The cytotoxic effect of two conventional antitumor antibiotics BLM and MMC and a new MMC analog KW-2149 was studied. These drugs share an important lung toxicity profile and all probably exert part of their cytotoxic effect by the generation of oxygen radicals.[39,59,60]

In a first set of experiments the oxygen radical production by human granulocytes was measured after exposure to different concentrations of MMC and BLM. Even after pre-stimulation with PMA, granulocytes failed to produce an increased amount of radicals. These results are in accordance with earlier work by Moseley *et al.*[18] These investigators also failed to detect increased superoxide production by BLM-exposed granulocytes. Only after these granulocytes had been incubated with a BLM-exposed lung explant was superoxide production increased. These results and our own data suggest no direct increase in oxygen radical production by granulocytes after drug exposure. Oxygen radical production by these blood cells might contribute at a later stage, after indirect stimulation. Such an indirect effect was also observed on separate occasions in one out of four patients in group C.

The direct stimulatory effect of the three different drugs, each tested at five concentrations and after an exposure of 24 h, on the production of three different cytokines was investigated in the second part of this study. TNF-α, TGF-β and IL-6 were measured in the supernatants of cell cultures of human macrophages, the human colorectal cell line DLD-1, and both HUVEC and HMEC. The 48 h incubation with BLM was shown to stimulate the confluent endothelial cell cultures to an increased production of TGF-β. This observation validates prior experiments.[61,62] This difference in endothelial cytokine production suggests a different mechanism of action for the mitomycins.

The direct effect of these different cytotoxic agents on human endothelial cells was investigated in the third part of our study. In order to differentiate between a dormant microvessel system such as in the normal lung and the tumor vasculature, cytotoxicity tests were performed both in confluent and semiconfluent cultures. We used both umbilical vein endothelial cells and immortalized endothelial cells. This was considered useful in order to control for possible differences between endothelial cells originating from different veins.

One conclusion is that all three agents are cytotoxic/cytostatic to endothelial cells at concentrations observed *in vivo*. The magnitude of this effect is dependent on the drug concentration, on the exposure time and ôn the proliferation state of the endothelial cells. Semiconfluent cultures were reproducibly more sensitive than confluent cultures, confirming the proliferation dependency of the cytotoxicity.

There have been several investigations reporting on the *in vitro* cytotoxic effect of BLM and MMC on different endothelial cell cultures.[40,45] Incubation of semiconfluent endothelial cultures with BLM for 18 h has been observed to result in increased chromium release. MMC; a drug with a potential for severe lung toxicity, was also shown to be cytotoxic for bovine capillary endothelial cells and may cause endothelial toxicity. The *in vitro* cytotoxicity of KW-2149 has only been reported previously for cancer cells. In this study we have shown that semiconfluent endothelial cells, supposed to mimic the cycling endothelial compartment associated with a growing tumor, are more sensitive than confluent cells. We have also shown that all three antitumor cause toxic effects to endothelial cells and this effect is not limited to cytostasis.

The endothelial morphology clearly demonstrated cytotoxic effects for all three agents. The difference in effect between confluent and non-confluent cultures of endothelial cells is especially pronounced for BLM. BLM exerts its toxic effect almost exclusively as a proliferation-dependent effect. This occurs at much lower concentrations when the time of incubation is prolonged. The confluent cultures seem to be resistant to this drug. The observation that BLM exerts time- and proliferation-dependent cytotoxic effects may provide an experimental reason for protocols with lower systemic plasma concentrations by employing the continuous infusion of the drug. Continuous exposure

to BLM may result in enhanced injury of the more sensitive, proliferating endothelial compartment in tumors, whereas the lower systemic concentration of the drug may result in reduced pulmonary toxicity. Non-proliferating endothelial cells are much more sensitive towards MMC and KW-2149 than to BLM. This suggests that the mitomycins exert an antivascular effect which is less dependent on proliferation.

Although semiconfluent cultures are more sensitive than confluent ones, the difference in proliferation plays only a minor role in the cytotoxic effect of these drugs. This implies that the vascular toxicity of MMC and KW-2149 cannot only be explained exclusively by their alkylating potential. The effect on endothelial cells is probably mediated by a mechanism different from the alkylating properties. The possible role of free radicals in the specific anti-endothelial effect of MMC and its analogs should be considered. Comparison of the studied drugs, based on their respective molecular weight, suggests KW-2149 as the most toxic agent for endothelial cells. This is not different for the *in vitro* cytotoxicity data against a variety of tumor cell lines. The cytotoxicity of BLM and MMC towards semiconfluent cultures seem to be comparable, at least in the concentration ranges achievable in patients. BLM is the least potent drug against non-proliferating endothelial cells. These *in vitro* observations match the clinical data on pulmonary complications. Important lung toxicity is related in a dose-dependent manner with KW-2149 administration. This can be explained by the *in vitro* cytotoxic effect starting to occur at very low concentrations for incubation times of 2 and 18 h. It might also reflect the fact that the toxicity of this drug related with both bolus injection and continuous infusion is nearly always mainly restricted to the lungs.

In conclusion, we have shown that three different antitumor antibiotics exert a dose- and proliferation-dependent cytotoxic effect on endothelial cells. The sensitivities of both types of endothelial cells are comparable to those obtained with a colon carcinoma cell line. These data have confirmed the superior activity of KW-2149 over MMC against the DLD-1 cell line and this superior cytotoxicity of KW-2149 over MMC was also observed with the endothelial cells. These data are in accordance with a possible contributing anti-angiogenic effect of these drugs in their antitumor effect *in vivo*. We failed to observe a distinct increase of oxygen radical production or cytokine production by any of the agents, with the sole exception of increased TGF-β production by endothelial cells after exposure to BLM. These data suggest that the mechanisms of lung toxicity for BLM and the mitomycins are at least in part dissimilar. The important cytotoxic effect on endothelial cells by the mitomycins and the known vascular toxic effects warrant further studies on the more discrete toxic effect exerted by the drugs on endothelial cells.

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