

Liposomal doxorubicin in the treatment of AIDS-associated Kaposi's sarcoma: clinical, histological and cell biological evaluation

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SUMMARY

AIDS-associated Kaposi's sarcoma (KS) in eight patients was treated with the systemic application of liposomal doxorubicin (20 mg/m² per cycle). After six cycles of treatment a significant regression of KS was observed in all patients. Tumour volume was reduced from 556 ± 635 mm³ before therapy to 42 ± 134 mm³ after therapy as determined by ultrasonography of selected tumours. Histological examination revealed a reduction of tumour-like structures and the absence of KS spindle cells in involved areas after therapy. *In vitro* experiments with KS-derived cell cultures, which most likely represent the KS spindle cells, suggested that liposomal doxorubicin may cause regression of KS via two different mechanisms: (i) by highly specific inhibition of KS spindle cell proliferation and (ii) by induction of monocyte chemoattractant protein-1 expression in KS spindle cells, which may result in increased recruitment of phagocytic cells (monocytes/macrophages) into the lesions. A cooperative action of both mechanisms may explain the high efficacy of liposomal doxorubicin in the treatment of AIDS-KS.

Key-words: AIDS, Kaposi's sarcoma, Doxorubicin, Liposome; Therapy, Spindle cells, Monocyte chemoattractant protein-1.

INTRODUCTION

Kaposi's sarcoma (KS) is the most common tumour associated with the acquired immunodeficiency syndrome (AIDS) and occurs in more than 25 % of all reported cases (Haverkos *et al.*, 1985;

Safai *et al.*, 1985). Histologically, KS lesions are characterized by prominent vascularization and numerous spindle-shaped cells. These so-called spindle cells are considered to be the tumour cells of KS (Ackerman and Gottlieb, 1988).

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Various forms of treatment of KS have been described. The appropriate treatment is selected according to the clinical presentation of the disease. Small, locally restricted lesions are treated with electrodesiccation and curettage, cryotherapy, radiotherapy, electron-beam therapy, or by surgical excision (Stickler and Friedman-Kien, 1991). More severe forms of the disease, presenting with multiple disseminated lesions and involvement of visceral organs, are treated with systemic chemotherapy (Stickler and Friedman-Kien, 1991). The agents used include doxorubicin, bleomycin, vinblastine, vincristine and etoposide (VP-16), employed either as monotherapy or in combination (Stickler and Friedman-Kien, 1991). Besides chemotherapy, biologic response modifiers such as interferon- α are systemically employed according to the hypothesis that these agents will prevent the immunosuppression following combination chemotherapy (Stickler and Friedman-Kien, 1991).

Until recently, all forms of systemic therapy of KS were hampered either by severe side effects (chemotherapy) or by low response rates (biologic response modifiers). Progress in chemotherapy was obtained by the availability of long-circulating "Stealth" liposomes loaded with doxorubicin. In an animal model it has been reported that the prolonged circulation time of liposomal doxorubicin accounts for the superior therapeutic effectiveness (Vaage *et al.*, 1992). Pharmacological data revealed a long plasma half-life, increased accumulation in tumour tissue and a decrease in uptake by tissues such as liver, spleen and bone marrow (Vaage *et al.*, 1992; Papahadjopoulos *et al.*, 1991; Baly *et al.*, 1990).

The objectives of our study were (i) to determine the efficacy of liposomal doxorubicin in the treatment of KS, (ii) to compare the histological presentation of KS before and after therapy and (iii) to investigate the molecular mechanisms which might account for the high efficacy of liposomal doxorubicin in the treatment of KS.

MATERIALS AND METHODS

Patients

Eight patients with biopsy-proven advanced AIDS-KS, presenting with progressive disseminated cutaneous lesions

including oedema of the face or limbs or oral lesions, were included in this study. Inclusion criteria were age ≥ 18 years, Karnofsky status $> 50\%$, white blood cell count $> 2,000/\mu\text{l}$, haemoglobin $> 10\text{ g/dl}$ and platelets $> 50,000/\mu\text{l}$. Exclusion criteria were acute opportunistic infections, non-Hodgkins lymphoma, systemic chemotherapy, radiation of KS within 4 weeks prior to entry into the trial, major psychiatric illness, cardiac failure and anthracycline intolerance. All patients gave written informed consent. The trial was approved by the institutional review board of the Medizinische Poliklinik, University of Munich, Germany.

The baseline evaluation consisted of a medical history and a physical examination. Indicator lesions were evaluated by sonographic measurements of tumour volume in all patients as described previously (Bogner *et al.*, 1993a and b). At least one indicator lesion accessible for cutaneous ultrasound examination was required if patients presented with oral or visceral lesions.

Clinical assessment and ultrasound measurements were performed at the end of each cycle within 48 h before administration of the next dose. Overall response was determined after six cycles of treatment.

KS-staging was performed according to the tumour/immune system/systemic illness (TIS) system as published by the AIDS Clinical Trials Group (ACTG) (Krown *et al.*, 1989).

Response criteria were applied as recommended by the ACTG (Krown *et al.*, 1989). A complete response (CR) was defined as the absence of any detectable residual disease, including tumour-associated oedema. In the case of remaining macular lesions, a biopsy documenting the histological absence of tumour-like structures was required.

A partial response (PR) was defined as either at least a 50% decrease in the sum of the areas of all previously existing lesions lasting for at least 4 weeks or at least a 75% decrease in nodularity of all previously existing lesions. The rating of PR was only permitted if no new skin or oral lesions appeared and if KS-related oedema did not worsen.

Stable disease (SD) was any response not meeting the criteria for CR, PR or progressive disease.

Progressive disease (PD) was defined as any occurrence of new lesions or the increase of more than 25% in the size of previously existing lesions, or the increase of oedema or effusions.

Treatment schedule

Liposomal encapsulated doxorubicin was administered intravenously at a dosage of 20 mg/m² every three weeks

AIDS = acquired immunodeficiency syndrome.
 AIDS-FIB = AIDS-derived fibroblast.
 AIDS-KS = AIDS-associated KS.
 FBS = foetal bovine serum.
 HSMC = human smooth muscle cell.

HUVEC = human umbilical vein endothelial cell.
 KS = Kaposi's sarcoma.
 KSC = AIDS-KS-derived cell culture.
 MCP-1 = monocyte chemoattractant protein-1.
 N-FIB = normal fibroblast.
 PAS = periodic acid and Schiff's reagent.
 TIS = tumour/immune system/systemic illness (system).

at the outpatient clinic of the Medizinische Poliklinik, University of Munich, Germany.

Liposomal doxorubicin (Stealth liposomal doxorubicin) was supplied by Liposome Technology Inc., Menlo Park, CA, USA.

Histological procedures

Skin punch biopsies were performed for histological evaluation of KS lesions. Immediately after removal, biopsies were transferred to a solution of freshly prepared 4 % paraformaldehyde in phosphate-buffered saline (PBS). The procedure for dehydration and paraffin-embedding was performed as described by Stürzl *et al.* (1992). Subsequently, haematoxylin-eosin, elastic van Gieson, PAS and prussian blue stained paraffin sections were examined.

Cell culture

AIDS-KS-derived cell cultures (KSC), AIDS-derived fibroblasts (AIDS-FIB), normal fibroblasts (N-FIB) and human smooth muscle cells (HSMC) were cultivated as described (Roth *et al.*, 1988). The following KSC were used in these studies: M7/3, M5/1, M8/2 and M8col4. AIDS-FIB were derived from an uninvolved area of the skin of the patients M7 and M5. N-FIB were derived from a skin biopsy of an HIV-negative man. Human umbilical vein endothelial cells (HUVEC) were purchased from Laevosan/Vienna and cultivated according to the instructions of the supplier. Suspension cultures of the myelomonocytic cell line U937 (ATCC, Wakersville, MD) were maintained in RPMI-1640 supplemented with 10 % FBS.

Proliferation assays

In order to analyse the inhibitory effect of liposomal doxorubicin on proliferation of cultivated cells, 3×10^4 cells of HSMC or 3×10^5 cells of KSC, AIDS-FIB, N-FIB and HUVEC were seeded into 24-multiwell plates and cultivated in the appropriate medium supplemented with 10 % FBS for five days (one day for HUVEC). After this incubation, all cultures reached confluency with an average cell number of 10^6 cells/well. Subsequently, cells were washed in DMEM and incubated in DMEM/10 % FBS with various concentrations of liposomal doxorubicin. Non-adherent U937 cells were seeded with an initial density of 10^6 cells/well in RPMI-1640 supplemented with 10 % FBS and various concentrations of liposomal doxorubicin. After 18 h of incubation with liposomal doxorubicin, $1 \mu\text{Ci}$ ^3H -thymidine was added to each well for 4 h. Finally, cells were harvested, and the incorporated radioactivity was determined by scintillation counting. Each experiment was carried out in triplicate and reproduced at least twice. The mean values were calculated.

RNA preparation and Northern blot analysis

Cells were grown to confluency, incubated for three days in DMEM/10 % FBS. Subsequently, the medium was replaced by fresh medium, and after 12 h of incubation, 10 ng/ml liposomal doxorubicin was added in

DMEM/10 % FBS or DMEM/0 % FBS. At different time points after the addition of liposomal doxorubicin, total RNA was isolated using the acid phenol method as described (Sambrook *et al.*, 1989). Isolated RNA was subjected to Northern analysis with a cDNA probe which was radioactively labelled using a random priming kit (Boehringer, Mannheim).

RESULTS

Eight patients were enrolled in a clinical study in order to examine the efficacy of liposomal doxorubicin in the treatment of AIDS-KS. The mean age of the patients was 39.6 ± 8.5 years. Classification of KS according to TIS stages (Krown *et al.*, 1989) was T1 (poor risk) and I1 (CD4 count below $200/\mu\text{l}$) in all patients, and S1 (opportunistic infections before KS) in 6 patients. Oral and/or gastrointestinal KS were present in 6 and 2 patients, respectively. One patient had proven pulmonary KS.

Skin punch biopsies were performed prior to therapy for histological evaluation of KS diagnosis (fig. 1A). Six cases showed typical histomorphological features of the plaque or the late plaque stage of KS. Two patients exhibited the criteria of an early nodular stage of KS. Numerous bizarre-shaped, thin-walled vessels (fig. 1A, white arrow) and the typical KS spindle cells (fig. 1A, black arrow), which were focally arranged in short fascicles, were obvious in all sections examined. Tumour volume was $556 \pm 635 \mu\text{l}$ (range 22 to 2,204) as determined by ultrasonography.

After six cycles of treatment, the average tumour volume was reduced to $42 \pm 134 \mu\text{l}$ (range 0 to 624; $p < 0.01$, paired *t* test). Response was rated by the criteria mentioned in "Materials and Methods". One patient showed complete response; partial response was observed in seven patients. No patients showed progression or stable disease while on therapy. Lesions that, prior to therapy, had been clinically and ultrasonographically proven to be KS plaque or nodular stage showed a histological picture completely different from that expected in such cases after treatment with liposomal doxorubicin. The typical KS spindle cells were no longer seen, neither in the upper (fig. 1B) nor in the deeper part of the epidermis (fig. 1C). Especially in the upper third of the dermis, there was a variable number of small vascular structures (fig. 1B), sometimes resembling an early patch KS lesion. In the deeper part of the dermis, abundant siderophages were most evident (fig. 1C). No evidence of necrosis or prominent haemorrhagia could be detected in any of the cases.

The clinical study reported above demonstrated the high efficacy of liposomal doxorubicin in the treatment of AIDS-KS. Only minor side effects were observed in the patients enrolled. In order to investigate whether the rarity of adverse effects may be

explained by an increased sensitivity of the KS spindle cells to liposomal doxorubicin in comparison with other cells, we performed proliferation assays *in vitro* (fig. 2). In these experiments similar concentrations of liposomal doxorubicin were used as were calculated to be present in the serum of KS patients during the treatment (10 ng/ml-1 µg/ml). At these concentrations, proliferation of KS-derived cells, which exhibit features similar to those of KS spindle cells *in vivo* (Stürzl *et al.*, 1992), was strongly inhibited (fig. 2A). Proliferation of endothelial cells (fig. 2D), monocytes (fig. 2E) and smooth muscle cells (fig. 2F) was clearly inhibited to a lesser extent. Only the proliferation of fibroblasts which were established either from biopsies of an uninvolved area of the skin of AIDS-KS patients (fig. 2B) or from healthy persons (fig. 2C) was inhibited with equal efficacy as was the proliferation of KS-derived cells. The differences in growth inhibition were greatest at a concentration of 10 ng/ml of liposomal doxorubicin (fig. 3). At this concentration, DNA synthesis of KS-derived cells (fig. 3, open square), normal fibroblasts (fig. 3, open circle) and AIDS fibroblasts (fig. 3, black square) was less than 29.8 % of the values obtained from the untreated controls. By contrast, DNA synthesis of monocytes (fig. 3, open triangle), human smooth muscle cells (fig. 3, black triangle) and human endothelial cells (fig. 3, black circle) was still more than 79 % of the untreated controls. The increased inhibitory effect (≥ 2.5 -fold) of liposomal doxorubicin on DNA synthesis of KS-derived cells and fibroblasts in comparison with its effect on other cell types may be an important reason for the high efficacy of liposomal doxorubicin in the treatment of KS.

The absence of KS spindle cells after the treatment of patients with liposomal doxorubicin, without detectable necrosis, suggested that phagocytotic cells (monocytes/macrophages) may be involved in tumour regression. Therefore, we examined the effect of liposomal doxorubicin on the expression of

monocyte chemoattractant protein-1 (MCP-1) by Northern analysis. Interestingly, expression of this monocyte chemoattractant is strongly induced in KS-derived cells between 0.5 and 8 h after exposure to liposomal doxorubicin in concentrations of 10 ng/ml (fig. 4). As shown above, monocytes are less sensitive to liposomal doxorubicin than are KS-derived cells (fig. 3). Therefore, during treatment of AIDS-KS with liposomal doxorubicin, monocytes may still be able to respond to MCP-1 expression of KS cells by increased migration into the tumour. Removal of cytostatic KS spindle cells by the phagocytotic capability of monocytes/macrophages may explain the absence of the KS spindle cells in KS lesions subsequent to treatment with liposomal doxorubicin (fig. 1).

DISCUSSION

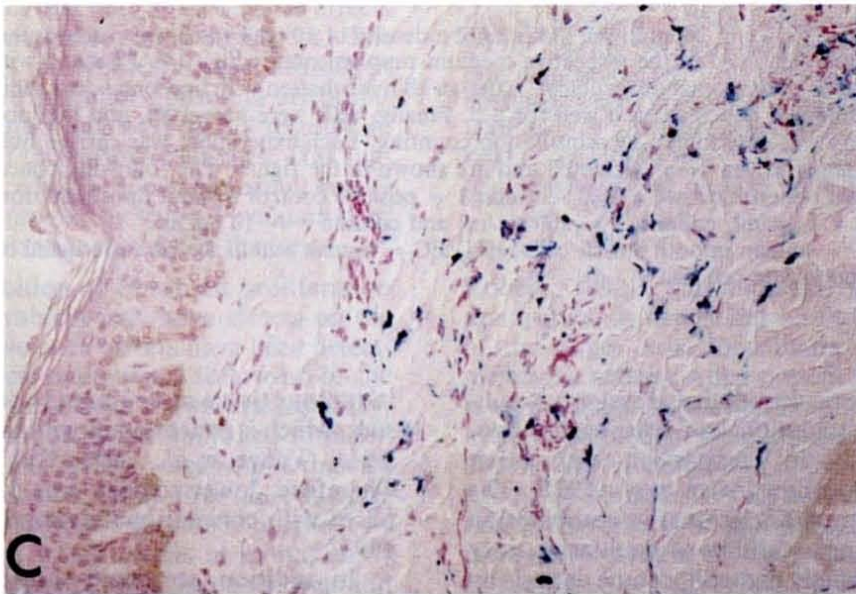
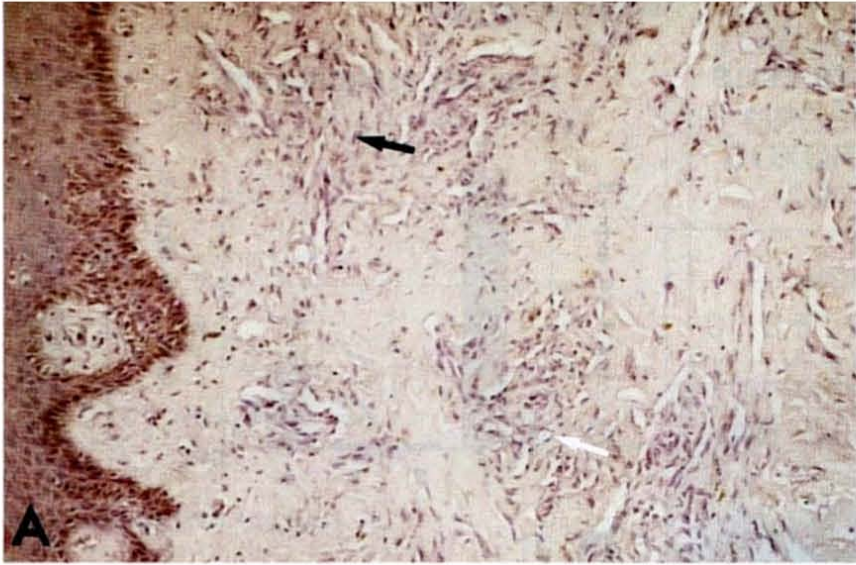
We report on a significant reduction of AIDS-KS lesions in eight patients after treatment with liposomal doxorubicin. Subsequent histological examination of areas where tumour regression was observed revealed an absence of KS spindle cells and a significant reduction of tumour-like structures. Adverse effects such as nausea, stomatitis and constipation, which were reported during therapy with free doxorubicin (Fischl *et al.*, 1993), were rare events during therapy with liposomal doxorubicin. None of the adverse effects was severe enough to terminate chemotherapy in the group of patients reported here. This indicates that liposomal doxorubicin is a powerful chemotherapeutic agent for the treatment of AIDS-KS.

Increased pharmacological efficacy of liposomal doxorubicin in comparison with free doxorubicin may explain the success of this treatment (Vaage *et al.*, 1992; Papahadjopoulos *et al.*, 1991; Bally *et al.*, 1990). Stealth liposomes, which were used in this study, reveal decreased uptake by the mononuclear

Fig. 1. Histological examination of KS lesions before and after treatment with liposomal doxorubicin.

A) Histological section of a KS biopsy before treatment with liposomal doxorubicin. Within this plaque of KS are an increased number of relatively small, sometimes dilated, bizarre-shaped, thin-walled vessels situated around pre-existing vascular structures in the entire reticular dermis (white arrow). These spaces are lined by inconspicuous endothelial cells without atypia, and there are also increased numbers of spindle cells (black arrow), monocytes/histiocytes and plasma cells (haematoxylin-eosin stain; magnification $\times 130$).

B and C) Histological section of a KS lesion after treatment with liposomal doxorubicin. The characteristic pattern of a KS plaque is no longer seen. Vascular structures, cleft-like spaces and spindle cells are reduced or absent (B: haematoxylin-eosin stain; magnification $\times 130$). Especially in the deeper part of the epidermis, abundant siderophages were seen. No evidence of necrosis or pronounced haemorrhage could be detected (C: iron stain with prussian blue, magnification $\times 130$).



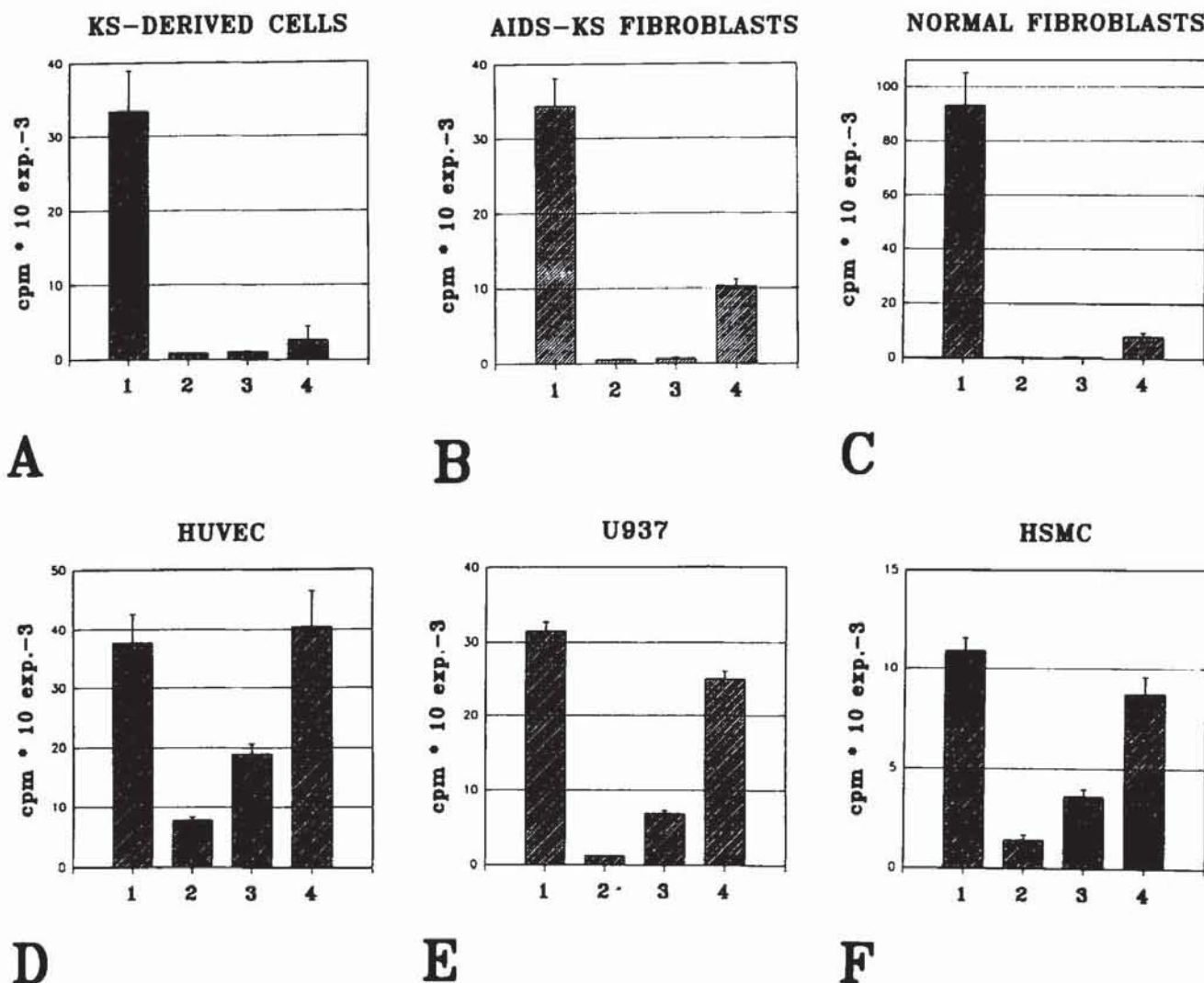


Fig. 2. Growth inhibition of different cell types by liposomal doxorubicin.

Cells were grown in 24-multiwell plates until a density of 10^6 cells/well was reached. Subsequently, cells were incubated in the respective medium supplemented with 10% FBS and with various concentrations of liposomal doxorubicin. After an 18-h incubation with liposomal doxorubicin, $1 \mu\text{Ci}$ ^3H -thymidine was added to each well for 4 h. Finally, cells were harvested, and the incorporated radioactivity was determined by scintillation counting. Each experiment was carried out in triplicate. The mean values were calculated and are shown in the figure. The following concentrations of liposomal doxorubicin were used: column 1 = positive control without liposomal doxorubicin, column 2 = $1 \mu\text{g/ml}$, column 3 = 100 ng/ml and column 4 = 10 ng/ml .

HSMC = human smooth muscle cells; HUVEC = human umbilical vein endothelial cells; U937 = myelomonocytic cell line.

phagocyte system (reticuloendothelial system), resulting in prolonged circulation half-lives of the liposomes in the body in comparison with other formulations of liposomes (Allen *et al.*, 1991). The high vascularization of KS lesions, in combination with an abnormal permeability of capillaries, may result in a proportionally higher liposome deposition

in tumour tissue as compared with that in other tissues, which is concomitant with increased drug deposition (Vaage *et al.*, 1992; Rahman *et al.*, 1990). Therefore, lower toxicity was anticipated as compared with conventional doxorubicin (Fischl *et al.*, 1993).

In addition, we could show in this study that

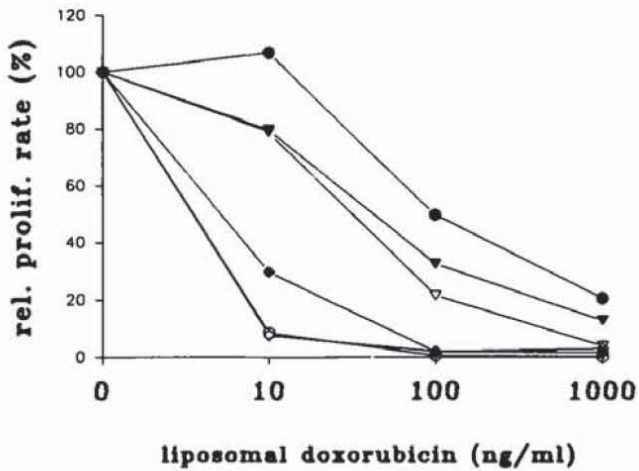


Fig. 3. Comparison of relative proliferation rates of different cell types under various concentrations of liposomal doxorubicin.

Relative proliferation rates of the respective cell types at different concentrations of liposomal doxorubicin in comparison with the untreated control were calculated. The difference in growth inhibition of the various cell types is greatest at a concentration of 10 ng/ml of liposomal doxorubicin. KS-derived cells, open square; normal fibroblasts, open circle; AIDS fibroblasts, black square; monocytes, open triangle; HSMC, black triangle; human endothelial cells, black circle.

liposomal doxorubicin inhibits the proliferation of KS-derived cells and fibroblasts more efficiently than it inhibits the proliferation of endothelial cells, human smooth muscle cells and monocytes. Increased specificity of liposomal doxorubicin for KS spindle cells in comparison with that for other cell types may contribute to the fact that only a few adverse effects were observed in patients during treatment. The difference in growth inhibition was most obvious at concentrations of 10 ng/ml of liposomal doxorubicin, whereas concentrations of 1 μ g/ml resulted in similar inhibition of the various cell types. This suggests that treatment with liposomal doxorubicin may be even further improved by careful evaluation of the optimal concentrations used for therapy.

The strong inhibition of fibroblast proliferation suggests that doxorubicin may have effects on the connective tissue. No such effects have been detected during histological and clinical follow-up of the therapy. However, it has been shown that anthracyclines, such as doxorubicin, are subjected to metabolic reduction leading to very reactive free radicals (Benčekroun and Robert, 1992). Free radicals cause membrane lipid peroxidation or induce breakage of the DNA backbone, which results in inhibition of proliferation of eukaryotic cells (Benčekroun and

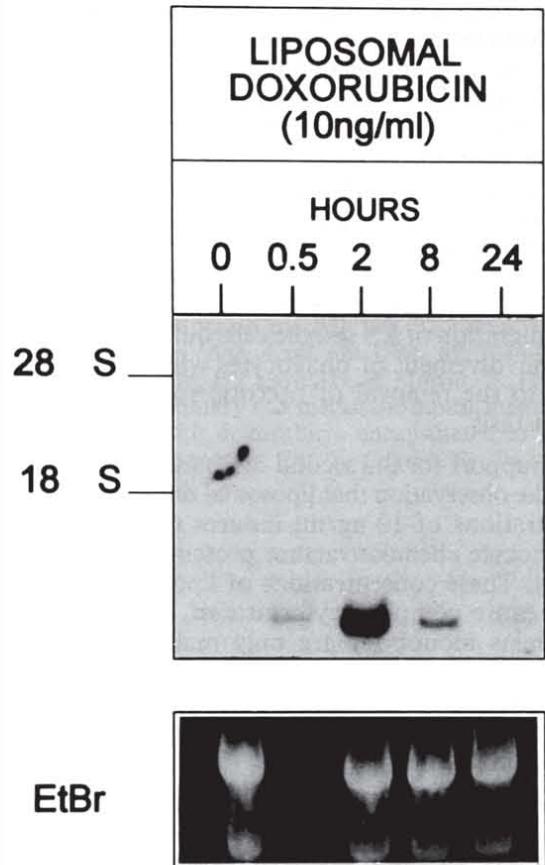


Fig. 4. Induction of monocyte chemoattractant protein-1 expression in KS-derived cells by liposomal doxorubicin.

KS-derived cells were grown to confluency, incubated for three days in DMEM/10 % FBS. Subsequently, the medium was replaced by fresh medium. After a 12-h incubation, 10 ng/ml liposomal doxorubicin was added in DMEM/10 % FBS, and RNA was isolated after 0, 0.5, 2, 8 and 24 h. Northern blot hybridization with a radioactively labelled cDNA probe (specific activity: 1×10^9 cpm/ μ g DNA) specific for MCP-1 revealed significant induction of MCP-1 expression. Similar results were obtained when liposomal doxorubicin was added in DMEM/0 % FBS (data not shown). An ethidium bromide stain of the blotted RNA is shown in order to demonstrate that equal amounts of RNA were applied.

Robert, 1992). This indicates that the action of doxorubicin is dependent on the metabolic activity of the target cells. Fibroblasts may have reduced metabolic activity when present in normal tissue *in vivo*, in comparison with the situation *in vitro* which leads to permanent stimulation. Reduced metabolic activity *in vivo* may result in lower doxorubicin sensitivity of fibroblasts. This may explain the fact that no adverse effects of liposomal doxorubicin on the connective tissue were recognized during treatment of patients.

The histological follow-up of therapeutic regression of KS lesions demonstrated the absence of the KS spindle cells in involved areas. Areas where lesions in a plaque or nodular stage were present before therapy revealed either significant reduction or complete absence of tumour-like structures. Necrosis or pronounced haemorrhagia were not observed. The removal of the KS spindle cells without detectable necrosis suggests that, besides the cytostatic effect of liposomal doxorubicin, further mechanisms may be involved in tumour regression during therapy. In this context two mechanisms can be imagined: (i) migration of KS spindle cells out of the lesion and (ii) involvement of phagocytes which may contribute to the removal of necrotic spindle cells under cytostasis.

Support for the second mechanism was obtained by the observation that liposomal doxorubicin in concentrations of 10 ng/ml induces the expression of monocyte chemoattractant protein-1 in KS-derived cells. These concentrations of liposomal doxorubicin cause complete cytostasis of KS-derived cells, whereas monocytes are only marginally affected. These data suggest that increased migration of phagocytotic cells (e.g. siderophages) may indeed contribute to KS regression during treatment with liposomal doxorubicin.

In summary, we provide evidence that KS spindle cells are more sensitive than other cells to the cytostatic effects of liposomal doxorubicin. Together with the increased drug delivery into KS lesions, which has been suggested by earlier reports, this finding may explain the high efficacy of liposomal doxorubicin in the treatment of AIDS-KS.

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La doxorubicine en liposomes dans le traitement du sarcome de Kaposi lié au SIDA: évaluation clinique, histologique et biologique cellulaire

Huit sujets atteints de sarcome de Kaposi (SK) associé au SIDA (SK-SIDA) ont été traités par des liposomes porteurs de doxorubicine, à raison de

20 mg/m²/perfusion. Après 6 perfusions administrées à des intervalles de 3 semaines, on observe une régression du sarcome chez tous les sujets. Le volume tumoral, évalué par ultrasons, est passé de 556 ± 635 à 42 ± 134 µl. L'analyse histologique révèle une réduction des structures de type tumoral et une absence de cellules SK fusiformes dans les zones tumorales en involution après ce traitement. L'étude *in vitro*, sur des cultures de SK, montre que deux mécanismes peuvent être impliqués dans l'action réductrice de la doxorubicine: (1) une forte inhibition spécifique de la prolifération des cellules fusiformes et (2) une induction de l'expression de la protéine-1 chimioattractante monocyttaire dans les cellules fusiformes du SK, pouvant accroître le recrutement des cellules phagocytaires (monocytes/macrophages) dans les lésions. Une synergie d'action de ces deux mécanismes peut expliquer la grande efficacité de la doxorubicine en liposomes dans le traitement du SK-SIDA.

Mots-clés: SIDA, Sarcome de Kaposi, Doxorubicine, Liposome; Traitement, Cellules fusiformes, Protéine-1 monocyttaire.

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