ELSEVIER

Biochemical Pharmacology 61 (2001) 119–127

Biochemical Pharmacology

Prevention of human smooth muscle cell proliferation without induction of apoptosis by the topoisomerase I inhibitor topotecan

Bernhard R. Brehm[a], Christina Bock[a], Sebastian Wesselborg[b], Steffen Pfeiffer[c], Stefan Schüler[c], Klaus Schulze-Osthoff[d,*]

[a]*Department of Cardiology, University of Tübingen, Tübingen, Germany*
[b]*Department of Gastroenterology, University of Tübingen, Tübingen, Germany*
[c]*Department of Cardiovascular Surgery, University of Dresden, Dresden, Germany*
[d]*Department of Immunology and Cell Biology, University of Münster, Münster, Germany*

Received 23 February 2000; accepted 2 June 2000

Abstract

Despite significant improvements in the treatment of atherosclerotic disease involving procedures such as angioplasty, bypass grafting, endartherectomy, or stent implantation, secondary failure due to late restenosis still occurs in 30–50% of individuals. Restenosis and later stages of atherosclerotic lesions arise from a complex series of fibroproliferative responses to vascular injury that are triggered by potent growth-regulatory molecules and finally result in vascular smooth muscle cell proliferation, migration, and neointima formation. The aim of this study was to investigate the antiproliferative effects of the topoisomerase I inhibitor topotecan on human arterial coronary smooth muscle cells. Following incubation of cells with different drug concentrations, mitotic indices were measured by bromodeoxyuridine incorporation, while cellular mitochondrial activity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Continuous incubation with topotecan for 7 days resulted in a complete and dose-dependent reduction of smooth muscle cell proliferation, and topotecan inhibited cell proliferation in the presence of growth factors as well. In contrast, mitochondrial activity was only partially decreased. Remarkably, although even short-term incubations for 20 min were sufficient to induce a long-lasting growth inhibition, topotecan did not induce apoptosis. Our results therefore suggest that, based on its drug profile, the topoisomerase I inhibitor topotecan may be a promising drug to inhibit restenosis occurring after coronary angioplasty with local devices.

Keywords: Human coronary arteries; Smooth muscle cells; Topotecan; DNA topoisomerase I; Proliferation

1. Introduction

Despite its wide acceptance, coronary angioplasty is limited by the fact that restenosis still occurs in 30 to 50% of all individuals [1]. In recent years, much has been learned about the mechanisms underlying restenosis, a condition that can be dissected into two distinct pathophysiological steps. The first step, recoil and remodeling, involves the mechanical collapse and constriction of the treated vessel. The second step, induction of neointima formation, is a proliferative response to injury that is mainly characterized by cell proliferation and matrix formation [2–5]. Restenosis after vascular injury results from the interdependent actions of the ensuing thrombosis, inflammation, smooth muscle cell accumulation, and liberation of potent growth factors including PDGF, bFGF, and others [6]. As a consequence, vascular smooth muscle cells dedifferentiate from a contractile phenotype to diverse cells expressing a variety of different gene products [7,8]. The process further involves the secretion of basement membrane-degrading matrix metalloproteinases, proliferation, chemotactic migration into the intima, and the secretion of a large extracellular matrix that forms the neointimal fibroproliferative lesion [9–14].

Although macrophages, T-cells, and endothelial cells

* Corresponding author. Tel. +49 251 835 2936; fax: +49 251 835 2250.

E-mail address: kso@uni-muenster.de (K. Schulze-Osthoff).

Abbreviations: PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; DAPI; diamidino-phenylindol-dihydrochloride; haCSMC, human arterial coronary smooth muscle cells; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and TGF, transforming growth factor.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00514-1

play important roles in the atherosclerotic process, the progression of the disease is determined by smooth muscle cell behavior, especially cell proliferation [7]. Phenotype expression and cell proliferation depend on the functional integrity of DNA replication, DNA repair, cell cycle progression, and gene expression. We therefore speculated that drugs affecting these nuclear events with proven therapeutic efficacy in human malignancies might be effective in preventing fibroproliferative alterations in response to intima injury. In the present study, we investigated whether topotecan, an antineoplastic agent, could inhibit smooth muscle cell growth. Topotecan, a semisynthetic, water-soluble derivative of camptothecin, is a potent inhibitor of DNA topoisomerase I with demonstrated clinical activity against various human tumors [15–18].

DNA topoisomerase I and II constitute a class of nuclear proteins that are regulated prior to and during inducible gene expression. DNA topoisomerase I relaxes torsionally strained (supercoiled) duplex DNA, thus enabling replication and transcription [19,20]. This is accomplished by a covalent adduct between topoisomerase I and DNA, which induces transient single-stranded DNA breaks, allowing the passage of the second DNA strand through the nick before resealing the DNA breaks [15,18–20]. Topoisomerase I can be activated in smooth muscle cells by either protein kinase C or cyclic adenosine monophosphate/cyclic guanosine monophosphate-dependent protein kinases [21]. Topoisomerase I inhibitors are S phase-specific drugs that bind and stabilize the adduct complex, resulting in DNA breaks that cannot be religated in the presence of the drug, which in turn results in inhibition of RNA synthesis [19,20].

In the present study, we investigated the effects of topotecan on the proliferation of human coronary smooth muscle cells. Incubations for 7 and up to 14 days were performed to determine whether any drug resistance developed over time. Furthermore, shorter incubation periods were investigated to evaluate whether a single application could inhibit smooth muscle cell proliferation. Studies on the effects of different growth factors on human smooth muscle cells were performed as a model for growth factor release that occurs after balloon angioplasty or stent implantation.

2. Materials and methods

2.1. Cell culture

Human coronary arteries were obtained from two patients with endstage dilated cardiomyopathy (without macroscopic signs of atherosclerosis) who were undergoing heart transplantation (approved by the local ethics committee). HaCSMCs were isolated from the left descending coronary artery and passaged according to previously described techniques [22]. The growth rate of the cells was identical between the two donors. HaCSMCs were grown in a mixture of Waymouth MB 752/1 medium and Ham's nutrient mixture F12 (1:1 v/v) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 U/mL of streptomycin (GIBCO BRL). Postconfluent haCSMC grew in a "hill and valley" growth pattern characteristic for smooth muscle cells. Smooth muscle origin was confirmed by immunocytochemical stainings using monoclonal antibodies against smooth muscle α-actin (Progen). Cells were fed every third day and only used at passage 3 to 7 for the experiments. Stainings with DAPI (Roche Molecular Diagnostics) were performed to exclude the possibility of contamination by mycoplasma.

2.2. Drug

Topotecan (9-amino-camptothecin) is a lipophilic substance that can diffuse into the cell within 10 min [19,20, 23]. It was kindly provided by Dr. H.J. Staab (Smith-Kline Beecham), dissolved in growth medium, and sterile-filtered to form a stock concentration of 10^{-3} M. Further test concentrations were made with growth medium as diluent. Anticancer activities of topotecan have been demonstrated against a number of tumor types including metastatic ovarian cancer, small cell lung cancer, refractory acute leukemia, different tumors of the gastrointestinal tract, and different pediatric tumors [24–28]. Clinical dosages vary between 0.4 to 22.5 mg/kg body weight, leading to systemic peak concentrations of 185 to 1068 μg/L [23]. *In vitro* topotecan has been shown to exert growth-inhibitory effects in a variety of different cancer cell types [29,30]. In all phase I and II studies, administration of topotecan was a safe and effective treatment. Given systemically, it can induce diarrhea, leukopenia, and thrombocytopenia comparable to other chemotherapeutic agents [26].

2.3. Proliferation and cytotoxicity assays

Cells were counted and seeded onto 96-well culture plates at a density of 20,000 cells/well. Incubation with topotecan (10^{-11} to 10^{-5} M) was started 24 hr later following cell attachment. After 2, 3, 4, 7, and 14 days, final cell numbers were measured by determining mitotic indices through the incorporation of BrdU (colorimetric cell proliferation ELISA, Roche Molecular Diagnostics). Cell viability was also determined by measuring the conversion of MTT using a commercial kit (Sigma-Aldrich-Chemie). The MTT test measures mitochondrial activity and serves as an indicator of the cellular metabolic rate. In order to determine cytotoxic effects, LDH released into supernatants was analyzed using a cytotoxicity test kit (Roche Molecular Diagnostics). All assays were carried out according to the manufacturers' recommendations.

2.4. Measurement of apoptosis

For determination of cell death, haCSMCs were seeded in 6-well plates and treated with topotecan (10^{-11} to 10^{-5} M) for 24 hr. The leakage of fragmented DNA from apoptotic nuclei was measured by the method of Nicoletti *et al*.

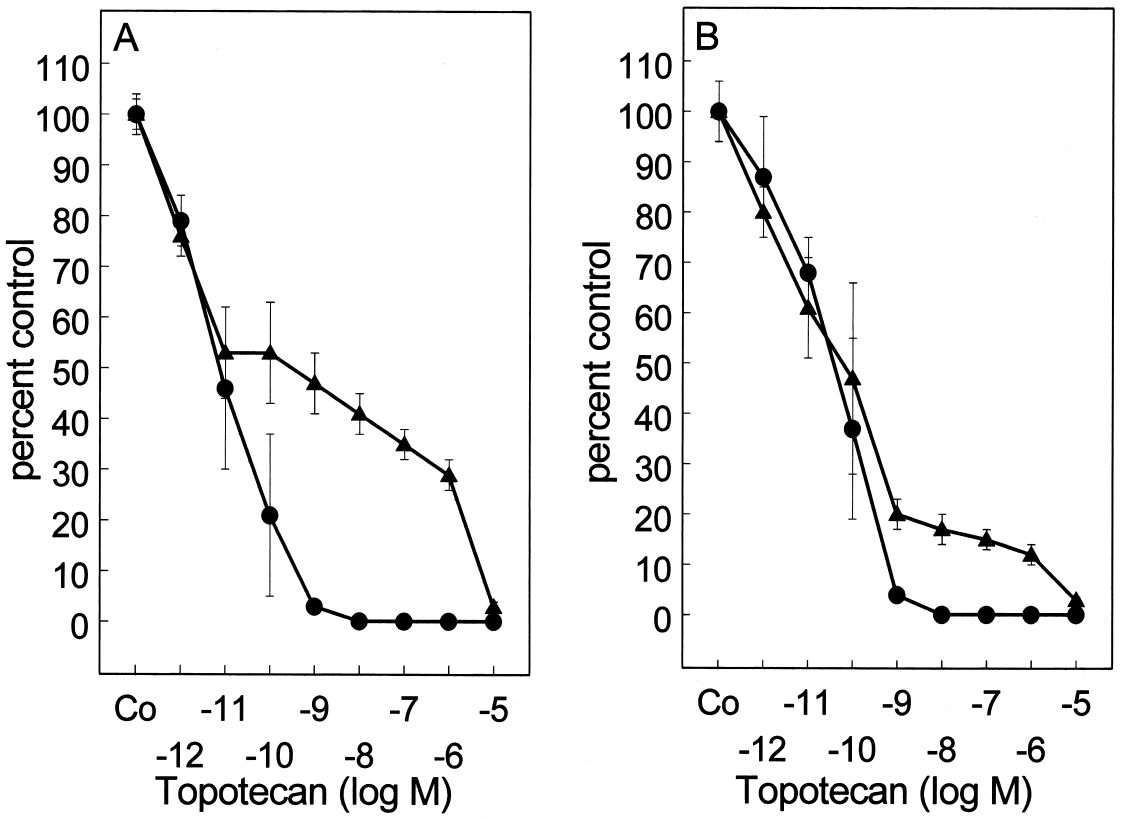

Fig. 1. Effects of topotecan on proliferation and mitochondrial activity in human arterial coronary smooth muscle cells. Cells were continously incubated for either 7 (A) or 14 days (B) with the indicated concentrations of topotecan. Proliferation was assessed by the BrdU-ELISA (●----●) and mitochondrial activity using the MTT test (▲----▲). Shown are the results ± SD as percent of the control (N = 6). After 7 days of drug incubation, the BrdU-ELISA showed a significant inhibition of proliferation, whereas the cells still contained considerable metabolic activity at a concentration of 10^{-6} M topotecan. Both proliferation and mitochondrial activity were reduced to similar concentrations after 14 days of incubation with topotecan.

[31]. Briefly, apoptotic nuclei were prepared by lysing cells in hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, 50 μg/mL of propidium iodide) and analyzed by flow cytometry. Nuclei to the left of the 2 N peak containing hypodiploid DNA were considered as apoptotic [32].

2.5. Growth factor assays

HaCSMCs were incubated in 10% fetal bovine serum with either 20 ng/mL of PDGF-AA, 20 ng/mL of PDGF-BB, 15 ng/mL of bFGF, or 10 ng/mL of TGF-β_1 (all from R&D Systems), before topotecan was added 24 hr later. Drug-containing medium was changed every third day. Growth factors were added every time the medium was replaced. Preliminary experiments showed that the concentrations of growth factors employed induced maximal stimulation of proliferation. The tests involved sixfold measurements of a range of topotecan concentrations (10^{-12} to 10^{-5} M) performed on three separate occasions under identical conditions.

2.6. Statistical analysis

Statistical analyses were performed using analysis of variance and Student's Newman–Keuls test for assessing significance. P values <0.05 were considered to denote statistically significant differences. Data are expressed as the means ± SD of 3 to 5 sets of experiments. The IC_{50} is defined as a growth rate corresponding to 50% of that observed under control conditions.

3. Results

3.1. Effect of topotecan on haCSMCs

To investigate the effects of topotecan on proliferation, we first measured the mitotic indices in haCSMCs using a BrdU-ELISA. After 7 days of continuous incubation, mitotic indices were dose-dependently reduced with an IC_{50} of 10^{-11} M and an IC_{max} of 10^{-8} M (Fig. 1A). Topotecan significantly inhibited haCSMC proliferation already at concentrations as low as 10^{-12} M (BrdU-ELISA control: 100 ± 3.0%, topotecan 10^{-12} M: 79 ± 5%, N = 6, $P < 0.005$). Only minimal proliferation was seen after treatment with 10^{-9} M topotecan. In contrast, the conversion of MTT was only decreased between 50% and 70% at concentrations between 10^{-11} and 10^{-6} M. Even at a high drug concentration of 10^{-6} M topotecan, the inhibition of metabolic

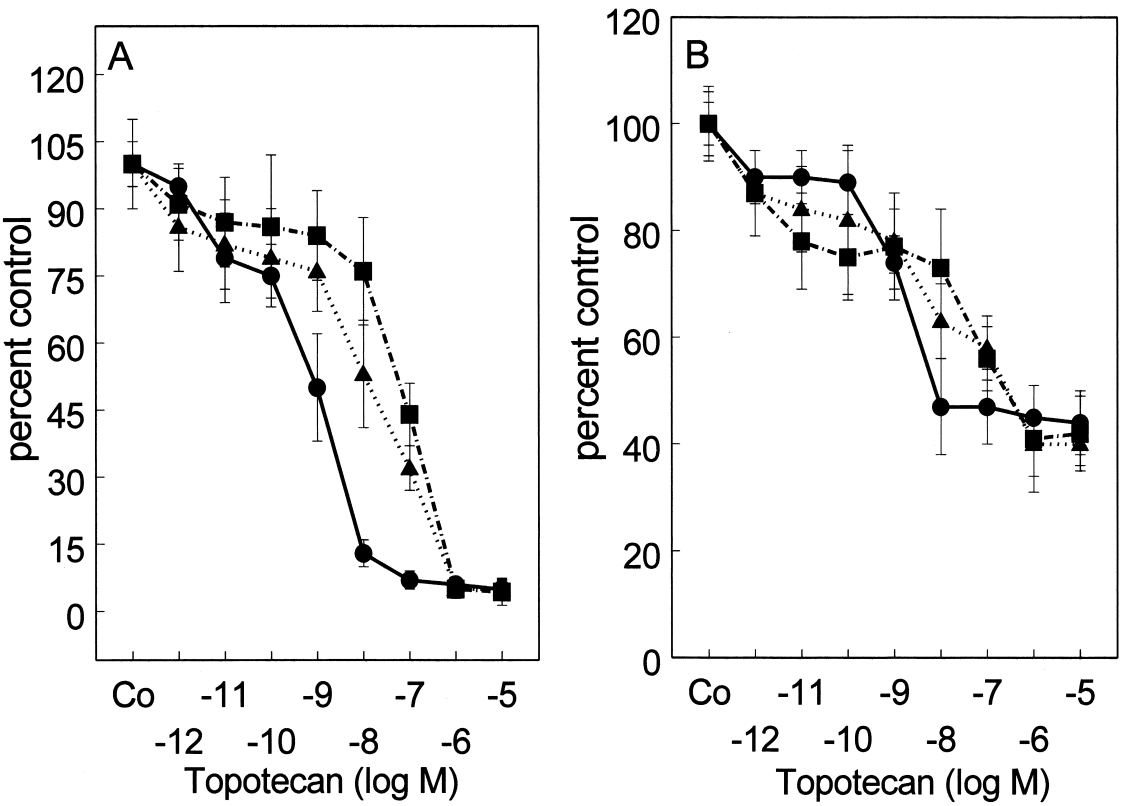

Fig. 2. Short-term incubations with topotecan. Incubations with different concentrations of topotecan were performed for either 24 hr (●----●), 6 hr (▲----▲), or 20 min (■----■) before the cells were further grown for an additional 7 days in the absence of the drug. Shown are the effects ± SD on proliferation (A) and mitochondrial activity (B). A rightward shift of the dose–response curves was observed that was dependent on the duration of drug incubation. At 10^{-6} M topotecan, proliferation was completely inhibited, whereas only a partial suppression of mitochondrial activity was observed.

activity did not exceed 70% of the control value. Prolonged application of topotecan for up to 14 days (Fig. 1B) resulted in a reduction of both MTT conversion and BrdU incorporation.

3.2. Short-term incubations with topotecan

Topotecan was also found to exert a dose-dependent antiproliferative effect when administered transiently for 24 hr, 6 hr, or even just 20 min before cell growth was continued for a further 7 days (Fig. 2A). Inhibition curves were slightly rightward-shifted compared to the previous results obtained with continuous incubations for several days (BrdU-ELISA, IC_{50} 24 hr 10^{-9} M, IC_{50} 6 hr 2×10^{-8} M, IC_{50} 20 min 8×10^{-8} M). Complete growth inhibition was obtained for all incubation periods at a concentration of 10^{-6} M. It is noteworthy that a single application for only 20 min was sufficient to decrease proliferation for at least 7 days and that no rebound effect was observed. The metabolic activity (Fig. 2B) was reduced by 55%. Application of topotecan for only 20 min prior to a further incubation for 14 days in the absence of the drug (data not shown) also failed to exert a rebound effect, while a complete inhibition of proliferation was induced by 10^{-6} M topotecan.

3.3. Effects of topotecan on growth factor-stimulated haCSMCs

Continuous incubation of haCSMCs with PDGF-BB (20 ng/mL) in the presence of 10% fetal bovine serum resulted in a significant growth stimulation after 7 days (163 ± 7% vs 100 ± 7% for controls, $P < 0.01$). bFGF (15 ng/mL) showed comparable growth-stimulating properties (157 ± 8% vs 100 ± 2% for controls, $P < 0.01$). Transient incubation of topotecan for 24 hr inhibited the growth factor-induced proliferation observed after 7 days dose-dependently. The respective IC_{50} values were 2×10^{-10} M for PDGF-BB and 3×10^{-10} M for bFGF, as measured by the incorporation of BrdU (Fig. 3A). PDGF-AA (20 ng/mL) and TGF-β_1 (10 ng/mL) stimulated proliferation of haCSMCs to 124 ± 4% and 125 ± 9%, respectively. Cell growth of PDGF-AA- and TGF-β_1-treated cells (Fig. 3B) was inhibited by topotecan in a dose-dependent manner (IC_{50} values after 7 days 4×10^{-10} M and 2×10^{-10} M, respectively). In the BrdU-ELISAs, proliferation was found to be arrested by topotecan, while MTT conversion was reduced by only 54% at the highest drug concentration. Thus, treatment with each of the four different growth factors produced significant increases in proliferation compared to controls. Nevertheless, topotecan significantly at-

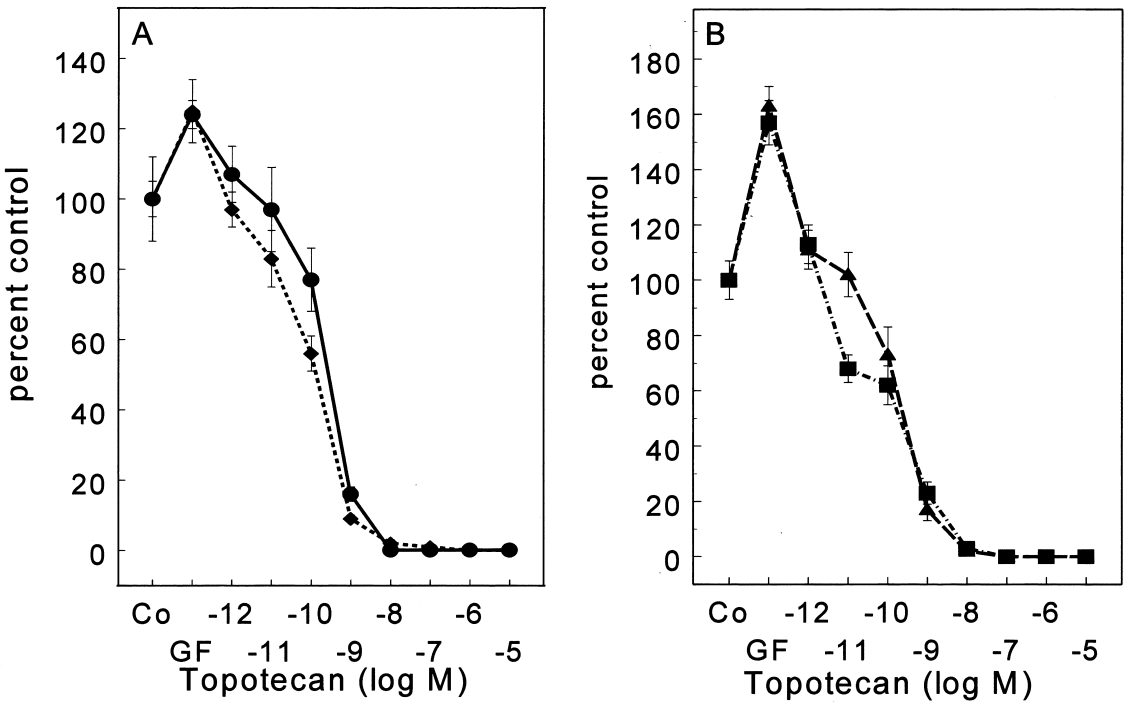

Fig. 3. Effect of topotecan on mitogenic stimulation induced by different growth factors (GF). Human coronary smooth muscle cells were incubated either in normal growth medium (Co) or in the presence of PDGF-AA (●----●, panel A). TGF-β_1 (◆----◆, panel A), PDGF-BB (▲----▲, panel B), or bFGF (■----■, panel B). Proliferation was measured after 7 days using the BrdU-ELISA (N = 6). The results show that topotecan also exerted a dose-dependent growth inhibitory effect in the presence of different growth factors.

tenuated haCSMC proliferation, indicating that the inhibition of cell growth induced by topotecan could not be overcome by mitogenic stimulation.

3.4. Cytotoxicity and determination of apoptosis

In order to determine whether growth inhibition by topotecan was due to overt cytotoxicity, we measured the release of LDH into culture supernatants. With drug incubations for 24 hr, 6 hr, and 20 min, no release of LDH was detectable after 3 days of culture (data not shown). Hence, over the concentration range used (10^{-12}–10^{-6} M), topotecan did not appear to induce a marked cytotoxic effect. In contrast, the proapoptotic agent staurosporine induced a strong increase (56%) in LDH liberation into the growth medium. Analysis of cell numbers after 2 days incubation at a concentration of 10^{-6} M revealed no reductions in cell counts (data not shown), indicating that topotecan was not cytotoxic even at concentrations found to be highly efficacious in the proliferation assays.

To further determine whether topotecan induced any cell death either by necrosis or apoptosis, FACS analyses using propidium iodide staining of hypodiploid DNA were performed. In the absence of the drug, about 6% of the cells underwent apoptosis, while application of topotecan at a concentration range between 10^{-11} and 10^{-6} M reduced proliferation, but did not increase apoptosis (Fig. 4A). Only the incubation with 10^{-5} M topotecan resulted in a minor increase in the apoptotic rate to 12%. As a positive control, staurosporine (10^{-5} M) induced apoptosis in 50% of smooth muscle cells, while the proportion of cells in the G1 phase decreased to 33% (Fig. 4, B and C). The effect of topotecan is also shown in DNA histograms (Fig. 4C), which demonstrate that unlike staurosporine topotecan did not induce apoptosis, although haCSMCs became arrested in the G2 phase of the cell cycle.

Since it was shown previously that topotecan does induce apoptosis [33], we investigated the drug effects in another cell line. Indeed, in leukemic Jurkat T-cells, marked apoptosis was already induced at a concentration of 10^{-9} M and detectable in 83% of the cells at 10^{-5} M topotecan (Fig. 4A). These data show that, while topotecan can induce apoptosis in cells predisposed to undergo apoptosis, it is not apoptotic for haCSMCs. Furthermore, staurosporine induced apoptosis in only 50% of haCSMC cells, while 92% of the Jurkat cells underwent apoptosis under these conditions (Fig. 4B).

Further microscopic evaluations revealed that topotecan did not trigger the appearance of morphological signs of apoptosis in haCSMCs such as cell rounding or detachment, confirming the absence of overt cytotoxicity (Fig. 5). Staining of the cells with the DNA dye DAPI showed that topotecan at concentrations of 10^{-6} and 10^{-5} M did not induce any significant chromatin condensation (Fig. 6, A and B), confirming its lack of a proapoptotic effect. In addition, immunocytochemical staining for α-actin revealed that topotecan (10^{-12} to 10^{-5} M) did not induce alterations in the normal filament arrangement (Fig. 6, C and D).

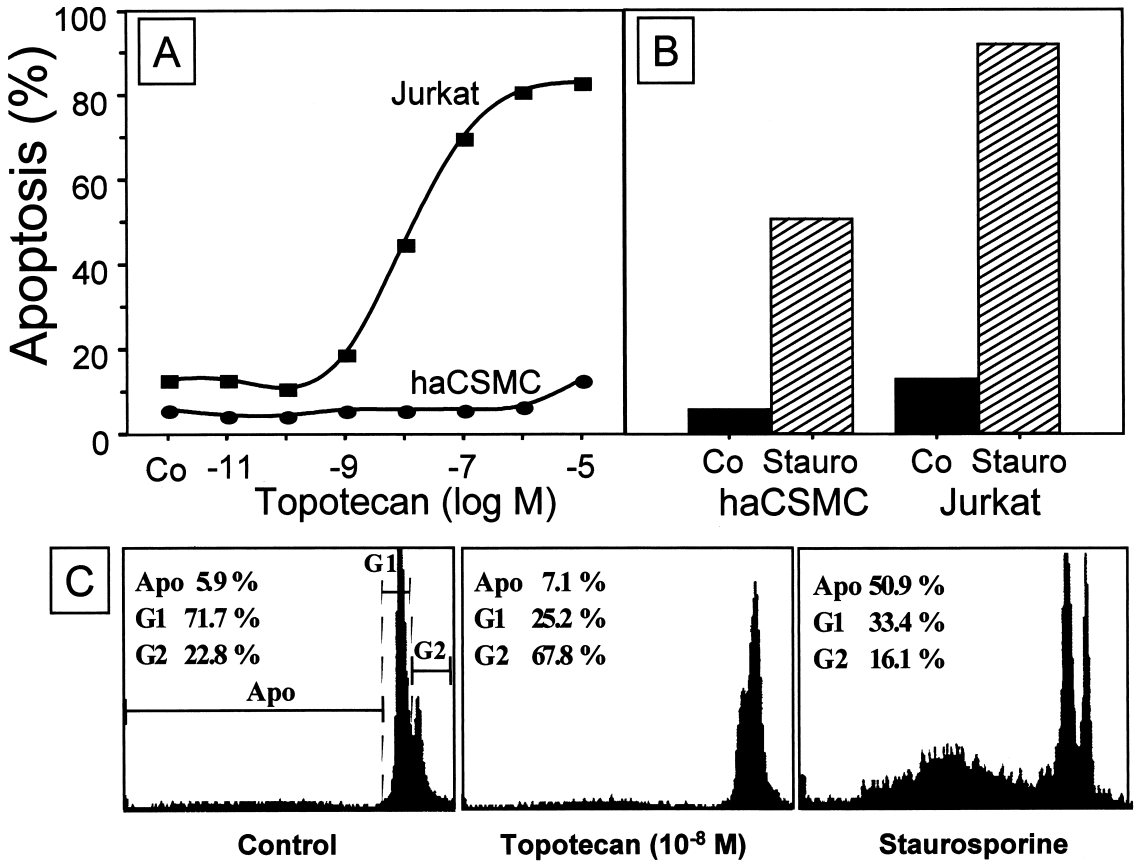

Fig. 4. Effect of topotecan on apoptosis. HaCSMCs and Jurkat cells were incubated with the indicated concentrations of topotecan (A) or 2.5 μM staurosporine (B). After 24 hr, induction of apoptosis was assessed by FACS analysis of hypodiploid DNA. Typical DNA histograms of the effect of topotecan and staurosporine in haCSMCs are shown in (C). A total of 8×10^3 nuclei was used to create each histogram. Peaks representing fragmented hypodiploid DNA (Apo) and G1 (G1) and S/G2 (G2) phases of the cell cycle are shown. The results demonstrate that, in contrast to haCSMCs, Jurkat cells were sensitive to topotecan-induced apoptosis.

4. Discussion

The present study shows that topotecan, an established inhibitor of DNA topoisomerase I, exerts potent and dose-dependent growth-inhibitory effects on proliferating human coronary smooth muscle cells. The antiproliferative effects were at least comparable to those reported in human colon or ovarian tumor cell lines [34]. This makes topotecan a promising candidate as a locally administered pharmaceutical agent to prevent restenosis after percutaneous translu-

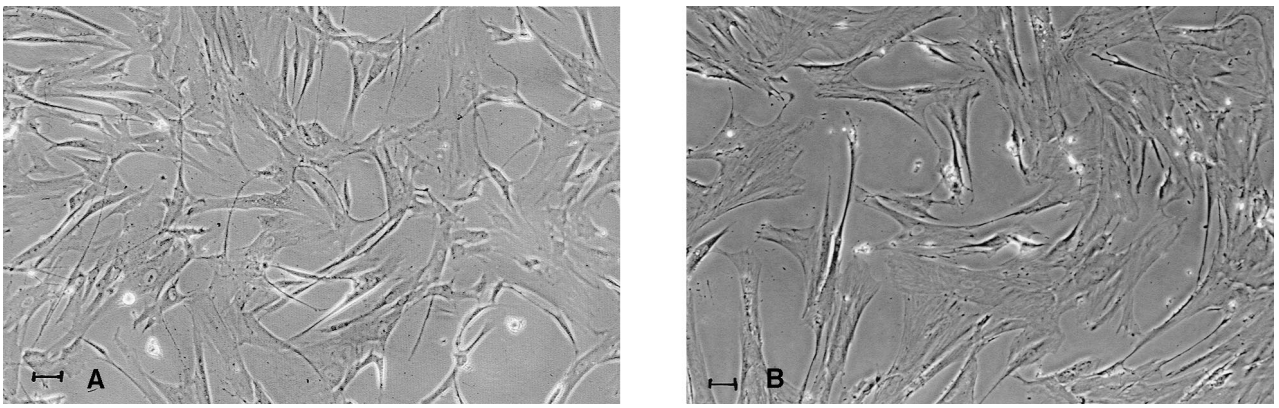

Fig. 5. Effect of topotecan on haCSMC morphology. (A) The phase-contrast micrograph shows untreated control cells. (B) Cultures incubated with 10^{-5} M topotecan revealed a decrease in cell numbers, while the cell shape was spindle-formed and the cytoplasm reduced. Dividing cells could not be detected. Scale bars = 0.2 mm.

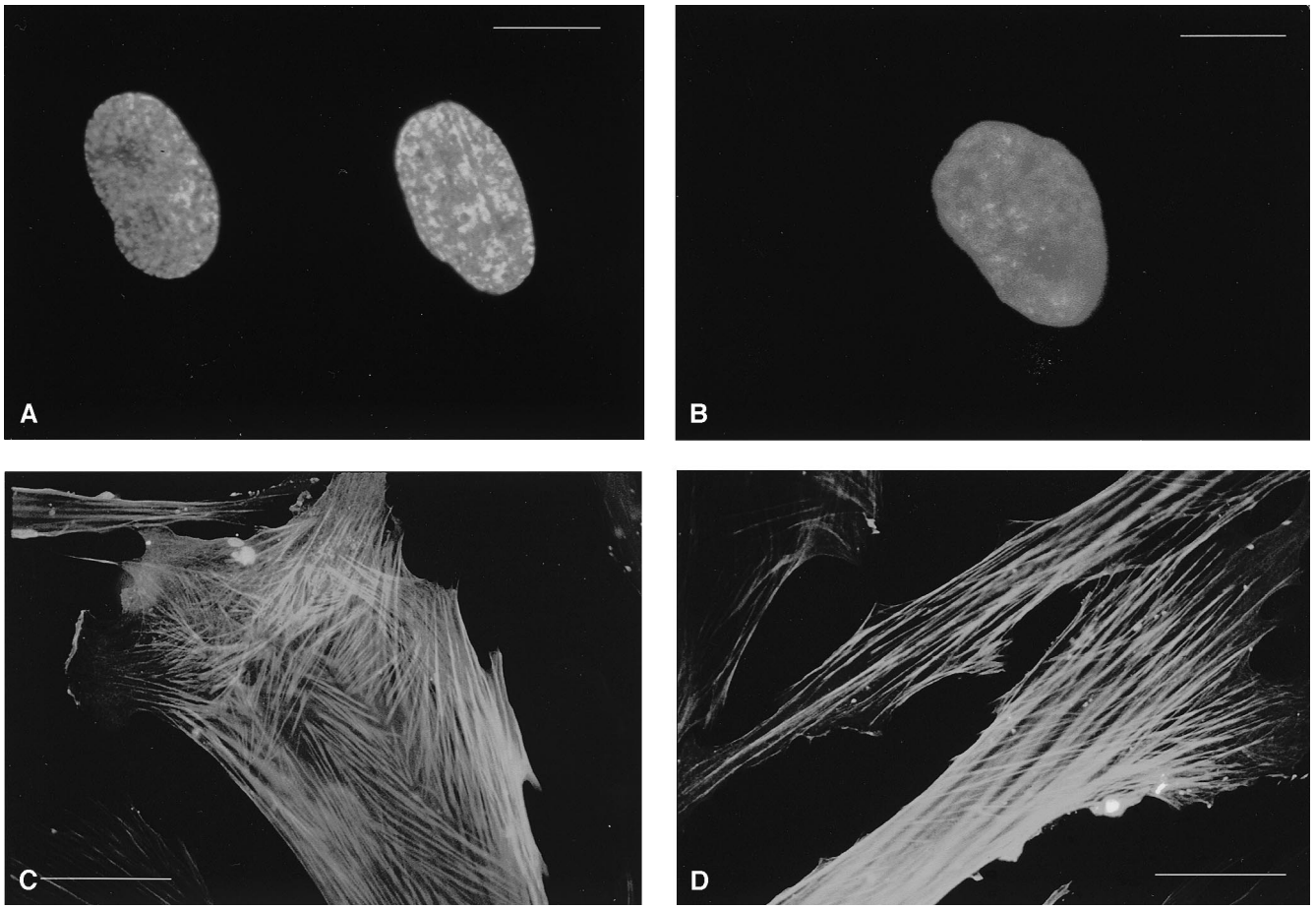

Fig. 6. Effect of topotecan on nuclear morphology and actin filament structure. Fluorescent microscopy of DAPI-stained nuclei. Both control cells (A) and cultures incubated for 7 days with 10^{-6} M topotecan (B) revealed normal nuclear morphology without any signs of chromatin condensation and DNA fragmentation. (C, D) Immunofluorescent micrographs. Cells were stained with an antibody against α-actin and a fluorescein isothiocyanate-labeled secondary antibody. Both untreated (C) and topotecan-treated cells (D) showed a normal arrangement of α-actin filaments. Scale bars = 20 μm.

minal angioplasty with local devices or coated drug-releasing stents. The antiproliferative activity of topotecan is characterized by its rapid cellular uptake and a sustained activity for a period of at least 14 days after an application for just 20 min to human coronary smooth muscle cells: even after such a short application, no rebound effect could be seen. Apoptosis was induced only at high doses of 10^{-5} M and only in approximately 10% of the cells. In stark contrast, apoptosis induced by topotecan in neoplastic Jurkat cells was pronounced, indicating that, unlike haCSMCs, Jurkat cells are predisposed to apoptosis.

It is noteworthy that no cytotoxicity or reduction in cell numbers could be detected after either 24 hr or 14 days of incubation with topotecan. Staining with DAPI, which allows the detection of chromatin condensation as an indicator for apoptosis, was only apparent in approximately 10% of cells when 10^{-5} M topotecan was used. Together with the flow cytometric measurements of DNA fragmentation, these results show that the growth-inhibitory effect of topotecan in haCSMCs up to a therapeutically relevant concentration of 10^{-6} M was not due to apoptosis. Indeed, human coronary arteries may be resistant to apoptosis, despite the fact that topotecan exerts a strong growth-inhibitory effect. It has also been reported that incubation of human umbilical venular endothelial cells with topotecan induced growth arrest without exerting a cytotoxic effect [35].

Whether tumor cells respond to topotecan seems to depend on cellular levels of p53. Gobert *et al*. showed that elevated expression of p53 increased the catalytic activity of topoisomerase I as measured by relaxation of supercoiled DNA [36]. Furthermore, it was reported that both p53-dependent and -independent mechanisms which can inhibit cell proliferation exist [37]. In atherosclerotic lesions from carotid arteries, elevated p53 protein levels were detectable in both smooth muscle cells and macrophages, and apoptosis occurred in only 3% of the cells as shown by terminal deoxynucleotide transferase-mediated dUTP nick end labeling [38]. Compared to atherosclerotically altered vessels, p53 protein is hardly detectable in the internal mammaria. Aoyagi *et al*. found high amounts of p53 in neointimal smooth muscle cells after arterial wall injury [39]. In malignant glioma cells, topotecan increases p53-dependent p21 protein levels and induces G2/M arrest without altering Bcl-2 or Bax protein levels or inducing apoptosis [40].

However, glioma cells were significantly sensitized to CD95 ligand-induced apoptosis by topotecan.

Experiments examining the mechanism of action of camptothecin and its derivatives showed that these drugs are immediate and profound inhibitors of DNA synthesis and cause DNA fragmentation in different cell types [41]. It can be assumed that one effect of topotecan in haCSMCs involves the inhibition of DNA replication. Topoisomerase I is not only involved in DNA replication processes during cell division, but is also enriched in active transcription units [42–45]. The observation that "topoisomerase I-cleavable complexes" occur preferentially within expressed genes underlines its involvement in mRNA synthesis [46, 47]. The topoisomerase I inhibitor camptothecin can inhibit RNA synthesis reversibly in cultured cells. The longer the incubation lasts, the more it becomes irreversible due to the dissociation of topoisomerase I-cleavable complex from transcription units [47]. Buckwalter *et al*. also showed that topoisomerase I is preferentially located in the nucleoli, but when topotecan was administered to the cells, the enzyme was translocated to non-nucleolar regions of the nucleus, and RNA synthesis was inhibited [48].

During angioplasty, thrombogenic factors and mitogens, such as growth factors, thrombin, ADP, and others, are released and in turn activate second messenger pathways involving protein kinase C or adenylate cyclase. Both pathways activate topoisomerases and subsequent RNA synthesis in vascular smooth muscle cells [49]. Thrombin and vasopressin increase topoisomerase I activity via different guanine nucleotide-binding proteins. Since a large number of genes become activated after vascular injury, topotecan-induced topoisomerase I inhibition has the potential to intervene at the level of RNA synthesis very early during initiation of growth factor expression, proliferation, and induction of matrix formation. It also acts in parallel on the DNA replication occurring before cell division.

In contrast to other antiproliferative substances, such as microtubuli-destabilizing agents [50], topoisomerase I inhibitors act mainly on cells in the S phase [51–53]. Cells in S phase are up to 1000-fold more sensitive to the cytotoxic effects of the topoisomerase I inhibitor camptothecin than cells in the G1 or G2 phase [52,54]. There is evidence that the regulation of topoisomerase I is altered in neoplastic cells: colon cancer cells contain five times as much topoisomerase I than their neighboring normal mucosal cells [55]. In addition, in proliferating mouse embryo fibroblasts, topoisomerase I activity is fourfold higher than in non-proliferating cells [56]. To our knowledge, topoisomerase I has not been studied previously in smooth muscle cells during proliferation or neointimal formation after vascular injury.

In summary, topotecan shows several features of a modern therapeutic drug for treating cardiovascular disease. The agent acts predominantly on proliferating cells, shows a potent growth-inhibitory effect in the presence of different mitogens, and induces only a mild degree of apoptosis in smooth muscle cells. A potential systemic genotoxic risk of topotecan for the organism could be limited by modern devices allowing a local drug application at the site of the stenotic lesion in the coronary artery. Thus, although our experiments with cultured human coronary smooth muscle cells derived from patients with dilated cardiomyopathy certainly may not fully reflect the pathological situation present *in vivo*, topotecan may be a substance with great promise for the inhibition of restenosis after coronary angioplasty or stent implantation.

References

[1] Popma JJ, Califf RM, Topol EJ. Clinical trials of restenosis after coronary angioplasty. Circulation 1991;84:1426–36.
[2] Kuntz RE, Gibson CM, Nobuyoshi M, Baim DS. Generalized model of restenosis after conventional balloon angioplasty, stenting and directional atherectomy. J Am Coll Cardiol 1993;21:15–25.
[3] Kuntz RE, Safian RD, Levine MJ, Reis GJ, Diver DJ, Baim DS. A novel approach to the analysis of restenosis after the use of three new coronary devices. J Am Coll Cardiol 1992;19:1493–9.
[4] Mintz GS, Popma JJ, Pichard AD, Kent KM, Satler LF, Wong C, Hong MK, Kovach JA, Leon MB. Arterial remodeling after coronary angioplasty: a serial intravascular ultrasound study. Circulation 1996; 94:35–43.
[5] Mintz GS, Popma JJ, Pichard AD, Kent KM, Salter LF, Chuang YC, Griffin J, Leon MB. Intravascular ultrasound predictors of restenosis after percutaneous transcatheter coronary revascularization. J Am Coll Cardiol 1996;27:1678–87.
[6] Ferrell M, Fuster V, Gold HK, Chesebro JH. A dilemma for the 1990s. Choosing appropriate experimental animal model for the prevention of restenosis. Circulation 1992;85:1630–1.
[7] Shanahan CM, Weissberg PL. Smooth muscle cell phenotypes in atherosclerotic lesions. Curr Opin Lipidol 1999;10:507–13.
[8] Sjölund M, Hedin U, Sejersen T, Heldin CH, Thyberg J. Arterial smooth muscle cells express platelet-derived growth factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. J Cell Biol 1988;106:403–13.
[9] Hanke H. Proliferative response of smooth muscle cells after experimental balloon angioplasty. Circulation 1996;93:202.
[10] Newby AC, Zaltsman AB. Molecular mechanisms in intimal hyperplasia. J Pathol 2000;190:300–9.
[11] Pauly RR, Passaniti A, Bilato C, Monticone R, Cheng L, Papadopoulos N, Gluzband YA, Smith L, Weinstein C, Lakatta EG, Crow MT. Migration of cultured vascular smooth muscle cells through a basement membrane barrier requires type IV collagenase activity and is inhibited by cellular differentiation. Circ Res 1994;75:41–54.
[12] Dartsch PC, Voisard R, Bauriedel G, Höfling B, Betz E. Growth characteristics and cytoskeletal organization of smooth muscle cells from human primary stenosing and restenosing lesions. Arteriosclerosis 1990;10:62–75.
[13] Reidy MA. A reassessment of endothelial injury and arterial lesion formation. Lab Invest 1985;53:513–20.
[14] Hanke H, Strohschneider T, Oberhoff M, Betz E, Karsch KR. Time course of smooth muscle proliferation in the intima and media of arteries following experimental angioblasty. Circ Res 1990;67:651–9.
[15] Liu LF. DNA topoisomerase poisons as antitumor drugs. Annu Rev Biochem 1989;58:351–75.
[16] Lackey K, Sternbach DD, Croom DK, Emerson DL, Evans MG, Leitner PL, Luzzio MJ, McIntyre G, Vuong A, Yates J, Besterman JM, Water soluble inhibitors of topoisomerase I: Quaternary salt derivatives of camptothecin. J Med Chem 1996;39:713–9.

[17] Gedik CM, Collins AR. Comparison of effects of fostriecin, novobiocin, and camptothecin, inhibitors of DNA topoisomerases, on DNA replication and repair in human cells. Nucleic Acids Res 1990;18:1007–13.
[18] Takimoto CH, Wright J, Arbuck SG. Clinical applications of the camptothecins. Biochim Biophys Acta 1998;1400:107–19.
[19] Pommier Y, Pourquier P, Fan Y, Strumberg D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim Biophys Acta 1998;1400:83–105.
[20] Verweij J. Topoisomerase I inhibitors and other new cytotoxic drugs. Eur J Cancer 1995;31A:828–30.
[21] Stewart L, Ireton GC, Parker LH, Madden KR, Champoux JJ. Biochemical and biophysical analyses of recombinant forms of human topoisomerase I. J Biol Chem 1996;271:7593–7601.
[22] Axel DI, Brehm BR, Wolburg-Buchholz K, Betz EL, Kövecker G, Karsch KR. Induction of cell-rich and lipid-rich plaques in a transfilter coculture system with human vascular cells. J Vasc Res 1996; 33:327–339.
[23] Herben VM, ten Bokkel Huinink WW, Beijnen JH. Clinical pharmacokinetics of topotecan. Clin Pharmacokinet 1996;31:85–102.
[24] Kudelka AP, Tresukosol D, Edwards CL, Freedman RS, Levenback C, Chantarawiroj P, Gonzalez de Leon C, Kim E, Madden T, Wallin B, Hord M, Verschraegen C, Raber M, Kavanagh JJ. Phase II study of intravenous topotecan as a 5-day infusion of refractory epithelial ovarian carcinoma. J Clin Oncol 1996;14:1552–7.
[25] Schiller JH, Kim K, Huston P, DeVore R, Glick J, Stewart J, Johnson D. Phase II study of topotecan in patients with extensive-stage small-cell carcinoma of the lung: An Eastern Cooperative Oncology Group Trial. J Clin Oncol 1996;14:2345–52.
[26] Rowinsky EK, Adjei A, Donehower RC, Gore SD, Jones RJ, Burke PJ, Cheng YC, Grochow LB, Kaufmann SH. Phase I and pharmacodynamic study of the topoisomerase I-inhibitor topotecan in patients with refractory acute leukemia. J Clin Oncol 1994;12:2193–2203.
[27] Verweij J, Lund B, Beijnen J, Planting A, de Boer-Dennert M, Koier I, Rosing H, Hansen H, Phase I and pharmacokinetics study of topotecan, a new topoisomerase I inhibitor. Ann Oncol 1993;4:673–8.
[28] Stewart CF, Baker SD, Heideman RL, Jones D, Crom WR, Pratt CB. Clinical pharmacodynamics of continuous infusion of topotecan in children: Systemic exposure predicts hematologic toxicity. J Clin Oncol 1994;12:1946–54.
[29] Kingsbury WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher G, Caranfa MJ, McCabe FL, Faucette LF, Johnson RK, Hertzberg RP. Synthesis of water-soluble (aminoalkyl)camptothecin analogues: Inhibition of topoisomerase I and antitumor activity. J Med Chem 1991;34:98–107.
[30] Kollmannsberger C, Mross K, Jakob A, Kanz L, Bokemeyer C. Topotecan—A novel topoisomerase I inhibitor: Pharmacology and clinical experience. Oncology 1999;56:1–12.
[31] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271–9.
[32] Wesselborg S, Engels IH, Rossmann E, Los M, Schulze-Osthoff K. Anticancer drugs induce caspase-8/FLICE activation and apoptosis in the absence of CD95 receptor/ligand interaction. Blood 1999;93:3053–63.
[33] Traganos F, Seiter K, Feldman E, Halicka HD, Darzynkiewicz Z. Induction of apoptosis by camptothecin and topotecan. Ann N Y Acad Sci 1996;803:101–10.
[34] Pizao PE, Smitskamp-Wilms E, Van Ark-Otte J, Beijnen JH, Peters GJ, Pinedo HM, Giaccone G. Antiproliferative activity of the topoisomerase I inhibitors topotecan and camptothecin on sub- and post-confluent tumor cell cultures. Biochem Pharmacol 1994;48:1145–54.
[35] Clements MK, Jones CB, Cumming M, Daoud SS. Antiangiogenetic potential of camptothecin and topotecan. Cancer Chemother Pharmacol 1999;44:411–6.
[36] Gobert C, Bracco L, Rossi F, Olivier M, Tazi J, Lavelle F, Larsen AK, Riou JF. Modulation of DNA topoisomerase I activity by p53. Biochemistry 1996;35:5778–86.
[37] McDonald AC, Brown R. Induction of p53-dependent and p53-independent cellular responses by topoisomerase I inhibitors. Br J Cancer 1998;78:745–51.
[38] Ihling C, Menzel G, Wellens E, Monting JS, Schaefer HE, Zeiher AM. Topographical association between the cyclin-dependent kinase inhibitor P21, p53 accumulation, and cellular proliferation in human atherosclerotic tissue. Arterioscler Thromb Vasc Biol 1997;17:2218–24.
[39] Aoyagi M, Yamamoto M, Azuma H, Nagashima G, Niimi Y, Hirakawa K, Yamamoto K. Expression of p53 protein and p53 gene transcripts in rabbit carotid arteries after balloon denudation. Histochem Cell Biol 1997;107:365–70.
[40] Winter S, Weller M. Potentiation of CD95L-induced apoptosis of human malignant glioma cells by topotecan involves inhibition of RNA synthesis but not changes in CD95 or CD95L protein expression. J Pharmacol Exp Ther 1998;286:1374–82.
[41] Tanizawa A, Fujimori A, Fujimori Y, Pommier Y. Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. J Natl Cancer Inst 1994;86:836–42.
[42] Fleischmann G, Pflugfelder G, Steiner EK, Javaherian K, Howard GC, Wang JC, Elgin SC. Drosophila DNA topoisomerase I is associated with transcriptionally active regions of the genome. Proc Natl Acad Sci USA 1984;81:6958–62.
[43] Muller M, Pfund W, Mehta V, Trask DK. Eukaryotic type I topoisomerase I is enriched in the nucleolus and catalytically active on ribosomal DNA. EMBO J 1985;4:1237–43.
[44] Gilmour DS, Elgin SC. Localization of specific topoisomerase I interaction within the transcribed region of active heat shock genes by using the inhibitor camptothecin. Mol Cell Biol 1987;7:141–8.
[45] Gilmour DS, Pflugfelder G, Wani JC. Topoisomerase I interacts with transcribed regions in Drosophila. Cell 1986;44:401–7.
[46] Stewart AF, Schütz G. Camptothecin-induced *in vivo* topoisomerase I cleavages in the transcriptionally active tyrosine aminotransferase gene. Cell 1987;50:1109–17.
[47] Zhang H, Wang I, Liu LF. Involvment of topoisomerase I in transcription of human ribosomal RNA genes. Proc Natl Acad Sci USA 1988;85:1060–4.
[48] Buckwalter CA, Lin AH, Tanizawa A, Pommier YG, Cheng YC, Kaufmann SH. RNA synthesis inhibitors alter the subnuclear distribution of DNA topoisomerase I. Cancer Res 1996;56:1674–81.
[49] Mattern MR, Nambi P, Bartus JO, Mirabelli CK, Crooke ST, Johnson RK. Regulation of topoisomerase I and II activities by cyclic nucleotide- and phospholipid-dependent protein kinases. Effects of interactions between the two transduction pathways. Receptor 1991;1: 181–90.
[50] Axel DI, Kunert W, Göggelmann C, Oberhoff M, Herdeg C, Küttner A, Wild DH, Brehm BR, Riessen R, Kövecker G, Karsch KR. Paclitaxel inhibits arterial smooth muscle cell proliferation and migration *in vitro* and *in vivo* using local drug delivery. Circulation 1997;96:636–45.
[51] Kessler M, Bosmann HB, Lohr R. Camptothecin effect on DNA synthesis in murine leukemia cells. Biochim Biophys Acta 1972;269: 210–6.
[52] Li LH, Fraser TJ, Olin EJ, Bhuyan BK. Action of camptothecin on mammalian cells in culture. Cancer Res 1972;32:2643–50.
[53] Horwitz SB, Horwitz MS. Effects of camptothecin on the breakage and repair of DNA during cell cycle. Cancer Res 1973;33:2834–6.
[54] Drewinko B, Freireich EJ, Gottlich JA. Lethal activity of camptothecin sodium on human lymphoma cells. Cancer Res 1974;34:747–50.
[55] Giovanella BC, Stehlin JS, Wall ME, Wani MC, Nicholas AW, Lui LF, Silber R, Potmesil M. DNA topoisomerase I-targeted chemotherapy in human colon cancer in xenografts. Science 1989;246:1046–8.
[56] Tricoli JV, Sahai BM, McCormick PJ, Jarlinski SJ, Bertram JS, Kowalski D. DNA topoisomerase I and II activities during cell proliferation and the cell cycle in cultured mouse embryo fibroblast (C3H 10T1/2) cells. Exp Cell Res 1985;158:1–14.