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Exposure of Human Vascular Smooth Muscle Cells to Raf-1 Antisense Oligodeoxynucleotides: Cellular Responses and Pharmacodynamic Implications

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ABSTRACT

To characterize the pharmacodynamic properties of CGP 69846A/ISIS 5132, an antisense oligodeoxynucleotide directed against the mitogenic signal transducer Raf-1 kinase, we investigated the elicited biological responses in human coronary artery smooth muscle cells. Cell exposure to CGP 69846A resulted in a reversible time- and concentration-dependent down-regulation of cellular Raf-1 gene expression and, ultimately, inhibition of cell cycle progression. The highest potencies of this compound to reduce Raf-1 mRNA and protein levels were observed after 24 and 48 hr of cell exposure, respectively, with corresponding IC_{50} values of ~100 and ~300 nM. Proliferation was inhibited with an IC_{50} value of ~300 nM after 72 hr. We interpreted the recovery rate of Raf-1 mRNA after cell exposure to antisense ODNs as the half-life ($t_{1/2}$ ~50 hr) of active intracellular CGP 69846A in our cell culture system. The endogenous Raf-1 turnover half-life of ~30 hr, as assessed by monitoring metabolically labeled Raf-1 protein, correlated kinetically with the antisense-induced protein decay rate (50% decay in ~33 hr), indicating that the efficiency of CGP 69846A in decreasing Raf-1 protein levels was rate-limited by the endogenous protein turnover rate. The pharmacodynamic effects of CGP 69846A antisense ODNs are therefore limited by the duration of its intracellular activity rather than by its ability to transiently decrease mRNA levels. Local steady state exposure to CGP 69846A may represent a new approach to prevent the transition of quiescent vascular smooth muscle cells into the pathologically hyperproliferating cells seen after angioplasty.

Accelerated growth of vascular smooth muscle cells is one of the major events of vascular proliferative disorders, including atherosclerosis and restenosis after angioplasty. A fundamental pathological feature of these disease states is the abnormal accumulation of cells within the neointimal space, resulting in neointimal lesion formation produced by alterations in the homeostatic balance between cell growth and cell death (Consigny, 1995; Haudenschild, 1995).

Studies in experimental animal models of vascular disease have identified several growth factors (e.g., platelet-derived growth factor, transforming growth factor β1, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor 1, angiotensin II, endothelin-1, tumor necrosis factor-α, and thrombin) that may play important pathogenic roles (Gibbons and Dzau, 1994; Ross, 1995; Schwartz *et al.*, 1995). Given the multiplicity of growth factors involved in neointima formation and the complexity of the disease process in the clinical context, it is unlikely that drugs targeted at single growth factors or their receptors will prove to be effective vehicles of vascular therapy. Recent studies indicate that growth factors activating the cellular MAPK signaling pathways may mediate antiapoptotic, growth-promoting signals, whereas growth factors activating the Jun kinase pathways may transmit proapoptotic stimuli (Verheij *et al.*, 1996; Xia *et al.*, 1995). Therefore, a more successful approach may be to target the components of these intracellular protein kinase signaling cascades that are shared by many growth-regulatory receptors.

ODNs offer the potential to block the expression of specific genes within cells. Inhibition of gene expression by an antisense ODN relies on the ability of the ODN to bind to a complementary mRNA sequence and decrease translation of the mRNA (Milligan, 1993). Local delivery of antisense ODNs directed against the expression of cell cycle regulatory genes, such as proliferating cell nuclear antigen, *cdc2*, *cdk2*, c-*myb*, and c-*myc*, inhibited neointima formation in several

ABBREVIATIONS: BrdU, bromodeoxyuridine; FBS, fetal bovine serum; G3PDH, glycerol-3-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; ODN, oligodeoxynucleotide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SmBM, smooth muscle cell basal labeling medium.

models of vascular lesion formation (Morishito *et al.*, 1993, 1994; Shi *et al.*, 1994; Simons *et al.*, 1992). Consequently, these encouraging pharmacological results highlight the need for a more detailed understanding of underlying antisense mechanisms.

The cytoplasmic Raf kinases are critical gatekeepers in growth factor signal transduction and oncogenic transformation because their activation initiates the MAPK signaling pathways. Three isotypes of Raf protein kinases have been identified in vertebrate cells: Raf-1, Raf-A, and Raf-B (Daum *et al.*, 1994; Davis, 1993; Storm *et al.*, 1990). The activation of these isoforms and, ultimately, activation of the cell cycle are suggested to be regulated differentially (Bogoyevitch *et al.*, 1995; Jaiswal *et al.*, 1994; Wu *et al.*, 1996). Recently, treatment of *in vivo* lung tumor xenografts with a phosphorothioate antisense ODN directed against Raf-1 kinase, CGP 69846A/ISIS 5132, was shown to inhibit gene expression and reduce tumor size progression (Monia *et al.*, 1996a). Furthermore, treatment of rat vascular cells with antisense ODNs targeted to Raf-1, Raf-A, or MAPK was shown to selectively reduce cell proliferation (Cioffi *et al.*, 1997; Robinson *et al.*, 1996).

We investigated the biological responses elicited by CGP 69846A in cultured human coronary artery smooth muscle cells. The antisense ODN was shown to specifically reduce, in a time- and concentration-dependent manner, the cellular levels of Raf-1 transcripts. This ultimately resulted in a significant suppression of cell proliferation by reducing the rate of cell cycle progression. The reduction of cellular Raf-1 protein levels after antisense treatment correlated kinetically with an endogenous Raf-1 protein turnover half-life of ~30 hr. The pharmacodynamic effects of CGP 69846A are therefore predicted by its duration of intracellular activity.

Materials and Methods

ODN synthesis. Synthesis and purification of phosphorothioate ODNs for tissue culture experiments were performed as described previously (Monia *et al.*, 1993).

Cell culture. Human coronary artery smooth muscle cells (passages 4–7; Clonetics, San Diego, CA) were grown in Falcon Primaria tissue culture flasks (75 cm^2/250 ml; Becton Dickinson, San Jose, CA) in Clonetics SmBM supplemented with 5% FBS, 0.5 ng/ml human recombinant epidermal growth factor, 5 μg/ml insulin, 2 ng/ml human recombinant fibroblast growth factor, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B at 37° in a 95% air/5% CO_2 humidified atmosphere. Cells were subcultured by aspiration of the growth medium followed by a 30-sec rinse with a solution containing 0.5 mM EDTA and 0.25 mg/ml trypsin.

Treatment of cells with ODNs. Cells were incubated with ODNs at a concentration of 10–400 nM in SmBM containing *N*-[1-(2,3-dioleoyloxy)-propyl]*N*,*N*,*N*-trimethylammonium chloride/dioleoyl phosphatidylethanolamine solution (Lipofectin; GIBCO BRL, Gaithersburg, MD) at a concentration of 0.25 mg/10 nM ODN, which corresponded to 0.175 mM *N*-[1-(2,3-dioleoyloxy)-propyl]*N*,*N*,*N*-trimethylammonium chloride and 0.175 mM dioleoyl phosphatidylethanolamine/10 nM ODN, or a 35:1 molar ratio of lipofectin to ODN. After 4 hr, the medium was removed and replaced with SmBM containing 5% FBS.

Northern blot analysis. The isolation of total RNA was achieved using the TRI system according to the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). Briefly, monolayer cell cultures were lysed directly in 60-mm culture dishes by the addition of 0.8 ml of TRI reagent supplemented with 8 μl of microcarrier gel. The lysate was mixed with 0.1 ml of 1-bromo-3-chloropropane and centrifuged at 12,000 × *g* for 15 min at 4°. Total RNA was precipitated from the separated aqueous phase by the addition of isopropanol, and the pellet was dissolved in stabilized formamide. RNA samples were quantified spectrophotometrically. Total RNA (5 μg) was fractionated by agarose-formaldehyde denaturing gel electrophoresis and transferred to nylon membranes (Hybond-N^+; Amersham, Arlington Heights, IL). Labeled Raf-1 cDNAs were synthesized *in vitro* in the presence of [α-^{32}P]dCTP (Amersham) from a human Raf-1 cDNA template (American Type Culture Collection, Rockville, MD) using random primers and Klenow enzyme (Prime-a-Gene; Promega, Madison, WI) and were used to probe the Northern blots. Hybridization analysis was carried out in Quickhyb solution (Stratagene, La Jolla, CA) at 65° and visualized by autoradiography. The blots were then stripped of radioactivity and reprobed with a ^{32}P-random-prime-labeled G3PDH cDNA probe (Clontech, Palo Alto, CA) to confirm equal loading. Raf-1 mRNA (3.6 kb) was quantified and normalized to G3PDH mRNA (1.4 kb) abundance using densitometric scanner analysis (Molecular Dynamics, Sunnyvale, CA) of autoradiograms exposed in the linear range of film density.

Western blot analysis. Cells were harvested in radioimmunoprecipitation buffer containing 20 mM Tris·HCl, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 1 mM dithiothreitol, 1% (v/v) Triton X-100, 100 kallikrein inactivating units/ml aprotinin, 1.0 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μM sodium molybdate, and 10 mM sodium fluoride. Lysates were cleared from particulate material by centrifugation at 10,000 × *g* for 10 min at 4°. Protein concentrations of cell lysates were determined according to the Bradford method (BioRad, Hercules, CA). Total cellular proteins (20 μg) were fractionated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and probed with anti-Raf-1 monoclonal antibodies (Transduction Laboratories, Lexington, KY) diluted in binding buffer at a concentration of 0.25 μg/ml. The binding buffer contained Tris-buffered saline, 1 mM EDTA, 0.1% (v/v) Tween 20, 2% (w/v) bovine serum albumin, 1 mM dithiothreitol, and 0.02% (w/v) sodium azide. Bound primary antibodies were detected with peroxidase-labeled secondary antibodies using the ECL method and autoradiography according to the protocol provided by the manufacturer (Amersham). The membranes were stripped of Raf-1 immunodetectors and reprobed with anti-GAPDH monoclonal antibodies (Advanced ImmunoChemical, Long Beach, CA). Raf-1 protein levels of 74 kDa were quantified and normalized to GAPDH protein (36 kDa) levels by densitometric scanner analysis (Molecular Dynamics) of autoradiograms.

Measurement of DNA synthesis and proliferation. Human smooth muscle cells, grown in Falcon Primaria 24-well microtiter plates (25,000 cells/well) to 80% confluence, were washed twice with phosphate-buffered saline and growth-arrested by replacing the media with SmBM devoid of serum and growth factors for 48 hr. Cells were then incubated with ODNs as described above. Cells were stimulated with 5% FBS for the indicated time periods and pulsed with [^{3}H]thymidine (5 μCi/ml, 83.2 Ci/mmol; New England Nuclear Research Products, Boston, MA) for the last 4 hr of this stimulation. The amount of radioactivity incorporated into trichloroacetic acid-insoluble material was counted using a Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD).

For proliferation experiments, smooth muscle cells, grown in 96-well Falcon Primaria microtiter plates (5000 cells/well) to 80% confluence, were washed twice with phosphate-buffered saline, and growth-arrested cells were incubated with ODNs as described above. The medium was removed from the wells and replaced with SmBM (without phenol red) containing 5% FBS in a final volume of 100 μl for the indicated time. Proliferation was assessed using the CellTiter Cell Proliferation Assay (Promega), which measures reduction in the tetrazolium compound MTS to formazan by viable cells. Briefly, 20 μl of MTS is added to each well during the last 3 hr of stimulation with mitogen, and the plates are returned to the 37° incubator. The

absorbance of formazan at 490 nm is recorded using a Ceres UV900C microplate reader (BioTek Instruments, Winooski, VT).

Flow cytometry analysis. Human smooth muscle cells, grown on Falcon Primaria 100-mm dishes to 80% confluence, were growth-arrested and treated with ODNs as described above. Cell cycle progression was monitored by incorporation of BrdU into newly synthesized DNA as described previously (Quelle *et al.*, 1993). The samples were analyzed with an EPICS Elite ESP flow cytometer (Coulter Electronics, Miami Lake, FL) using the 488-nm line of an argon laser and optical emission filters of 525 nm for BrdU. Bitmap gating on histograms of integral versus peak fluorescence signals was used to exclude doublets. The resulting DNA distributions were analyzed using Elite Software (v. 4.11; Coulter Electronics).

Metabolic turnover rate determination of Raf-1 protein. Human coronary artery smooth muscle cells were grown to 40% confluence and washed twice with methionine/cysteine-free medium. The cells were incubated with 0.4 mCi/ml [^{35}S]methionine/^{35}S-cysteine labeling mix (New England Nuclear) in medium devoid of methionine and cysteine for 2 hr. After the addition of an equal volume of 10% FBS in SmBM, the incubation was extended to 16 hr. After replacement with 5% FBS in SmBM, cell lysates were prepared after various time periods as described in Western blot analysis. Total cell lysates were incubated for 3 hr at 4° with 50 μl of agarose-conjugated anti-Raf-1 rabbit polyclonal antiserum or an unrelated control antiserum (anti-Rho B) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunocomplexes were collected by centrifugation, washed twice with radioimmunoprecipitation buffer, subjected to SDS-PAGE, and transferred onto nitrocellulose membranes (Bio-Rad). Radioactivity was quantified by phosphor-imaging using a Storm system (Molecular Dynamics).

Regression analysis. The normalized densitometric scanner data from Northern and Western blot autoradiograms as well as the data from the proliferation assay were fitted to a four-parameter first-order logistic equation by nonlinear regression using Prism software (GraphPAD Software, San Diego, CA) to calculate IC_{50} values. The PhosphorImager-quantified protein turnover data were subjected to a one-phase exponential decay equation by nonlinear regression analysis to calculate half-time values.

Results

CGP 69846A specifically reduces Raf-1 transcript and protein levels in human coronary artery smooth muscle cells. CGP 69846A/ISIS 5132, a phosphorothioate ODN of 20 bases in length that targets the 3′-untranslated sequence of Raf-1 mRNA, has been shown to reduce Raf-1 mRNA levels in human tumor cells. In contrast, a mismatched control analogue of CGP 69846A that contains seven base changes within the CGP 69846A sequence was found to be ineffective in reducing Raf-1 mRNA levels (Monia *et al.*, 1996a, 1996b).

To assess the pharmacological activity of these compounds in human vascular cells, we analyzed cellular Raf-1 mRNA and protein levels after ODN treatment. Growth-arrested human coronary artery smooth muscle cells were exposed to different concentrations of CGP 69846A or the mismatched control ODN in the presence of lipofectin. Lipofectin is a cationic lipid formulation that has been shown to increase cellular uptake of ODNs (Bennett *et al.*, 1992). The cells were subsequently re-exposed to growth medium and harvested at 24, 48, and 72 hr to assess time- and concentration-dependent effects of CGP 69846A. The normalized densitometric Raf-1 mRNA and protein values were fitted to a general sigmoidal dose-response equation by nonlinear regression analysis to calculate approximate IC_{50} values (Fig. 1). Raf-1 mRNA levels decreased in a concentration-dependent manner at all three time points. At the 24-hr time point, an IC_{50} value of ~100 nM was calculated for the CGP 69846A treatment (Fig. 1A). The ability of the antisense ODN compound to reduce Raf-1 mRNA levels subsequently diminished with IC_{50} values of ~150 nM at 48 hr and ~500 nM at 72 hr (Fig. 1, B and C, respectively). In comparison, the effect of CGP 69846A on Raf-1 protein expression was delayed in onset and of shorter duration. At 24 hr, CGP 69846A elicited a weak response (IC_{50} ~ 700 nM) that increased at 48 hr (IC_{50} ~ 300 nM) and diminished again after 72 hr (IC_{50} ~ 700 nM). Accordingly, the Raf-1 protein levels at these time points dropped by ~35%, ~65%, and ~40%, respectively, compared with mismatch ODN-treated cells. The mismatch control compound failed to affect Raf-1 mRNA and protein levels at these time points and concentrations, demonstrating the sequence-specific mode of action for CGP 69846A.

Application of CGP 69846A reduced proliferation of human coronary artery smooth muscle cells. To determine the effect of decreased Raf-1 gene expression on cellular proliferation, human coronary artery smooth muscle cells were incubated in the presence of lipofectin with either CGP 69846A or control mismatch ODNs, and the antiproliferative effects were measured using a colorimetric enzyme assay. The assay detects cells that have the ability to convert a tetrazolium dye into a formazan product through the action of NADPH-generating dehydrogenases. Stimulation of growth-arrested cells with serum for up to 96 hr resulted in a time-dependent, logarithmic increase in cellular proliferation (Fig. 2A). However, exposure of cells to 400 nM CGP 69846A before stimulation with serum resulted in a marked inhibition of cellular proliferation. The inhibitory effect of Raf-1 antisense treatment was not detectable at 24 hr; however, after 48 hr, an 30 ± 18% reduction in formazan formation was observed compared with untreated cell samples. At 72 hr, the effect increased to 48 ± 10% but diminished to 30 ± 12% after 96 hr. The mismatch ODN exhibited no significant effect on cell proliferation (Fig. 2A). The antiproliferative potency of CGP 69846A was assessed 72 hr after treatment of cells with increasing concentrations of ODN. CGP 69846A significantly diminished serum-induced proliferation in a dose-dependent manner with an IC_{50} value of 280 ± 40 nM (Fig. 2B). Again, serum-induced proliferation was not significantly altered in cells pretreated with control mismatch ODN.

CGP 69846A reduced the human coronary artery smooth muscle cell population in the DNA synthesis phase of the cell cycle. To identify the mechanism of the ODN-mediated inhibition of cellular proliferation, the effect of CGP 69846A on DNA synthesis was determined by flow cytometry. Cells were incubated with BrdU and treated with 400 nM CGP 69846A followed by growth stimulation with serum. Flow cytometry analysis revealed that in cells pretreated with CGP 69846A, there was a 31% reduction in the proportion of cells in S phase (cell population in S phase, 18.1%) compared with cells treated with lipofectin only (cell population in S phase, 26.4%), indicating that CGP 69846A reduced the rate of cell cycle progression.

To confirm the cell cycle analysis assessed by flow cytometry, [^{3}H]thymidine incorporation was measured at various time points after treatment of cells with 400 nM CGP 69846A (Fig. 3). Serum stimulation of growth-arrested, untreated

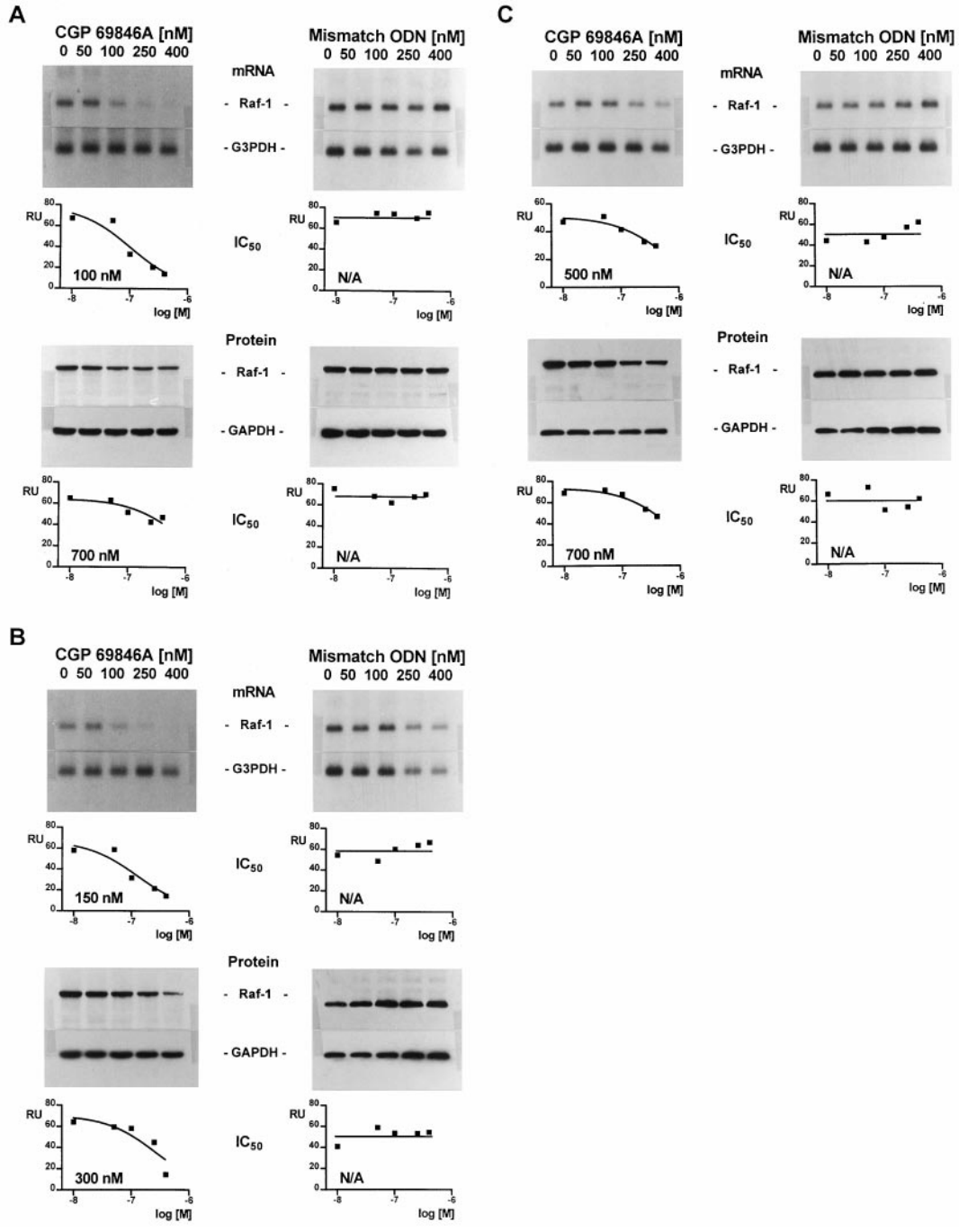

Fig. 1. Dose-response analysis of Raf-1 mRNA and protein expression in human coronary artery smooth muscle cells after treatment with CGP 69846A and mismatched control ODN. Growth-arrested cells were treated with the indicated concentrations of antisense ODNs in the presence of cationic lipid and analyzed for mRNA and protein expression by Northern and Western blotting, respectively. Cellular mRNA abundance of Raf-1 and G3PDH as well as Raf-1 and GAPDH protein levels were assessed 24 (A), 48 (B), and 72 (C) hr after antisense treatment. The normalized quantitative Raf-1 mRNA and protein units, indicated as relative units (*RU*), were analyzed by nonlinear regression to calculate IC_{50} values for CGP 69846A. IC_{50} values for the mismatch control ODNs were not applicable (*N/A*). Data shown were obtained from one of two similar experiments.

cells induced a time-dependent increase in the incorporation of labeled thymidine that peaked after 24 hr with a 3.7-fold maximal increase over basal levels in unstimulated cells. Exposure of growth-arrested cells to CGP 69846A before serum stimulation resulted in a 20 ± 17%, 35 ± 12%, and 40 ± 9% reduction in [^{3}H]thymidine incorporation as measured after 18, 24, and 48 hr, respectively.

Our cell treatment protocol did not induce any apoptotic or necrotic events in the presence of 400 nM CGP 69846A antisense ODN concentration for up to 4 days as analyzed by biochemical and morphological changes. No occurrence of apoptotic DNA fragmentation was observed, as assessed by enyzme-linked immunosorbent assay detection of BrdU-labeled DNA fragments from genomic DNA preparations obtained from antisense treated and untreated cells. In addition, cell viability and apoptotic indices were quantified by fluorescent microscopy using the DNA binding dyes acridine orange and ethidium bromide (data not shown).

The pharmacodynamic activity of CGP 69846A on cellular Raf-1 expression was reversible and could be extended by repeated applications. To monitor the extent and duration of CGP 69846A activity on cellular steady state Raf-1 transcript and translate expression levels, a time course study was performed over 5 day (Fig. 4). Exposure of cells to 400 nM CGP 69846A induced a rapid decrease in Raf-1 mRNA abundance; at the end of a treatment period of 4 hr, the level dropped by 70% and 24 hr later by 80% compared with untreated cells (Fig. 4A). Subsequently, Raf-1

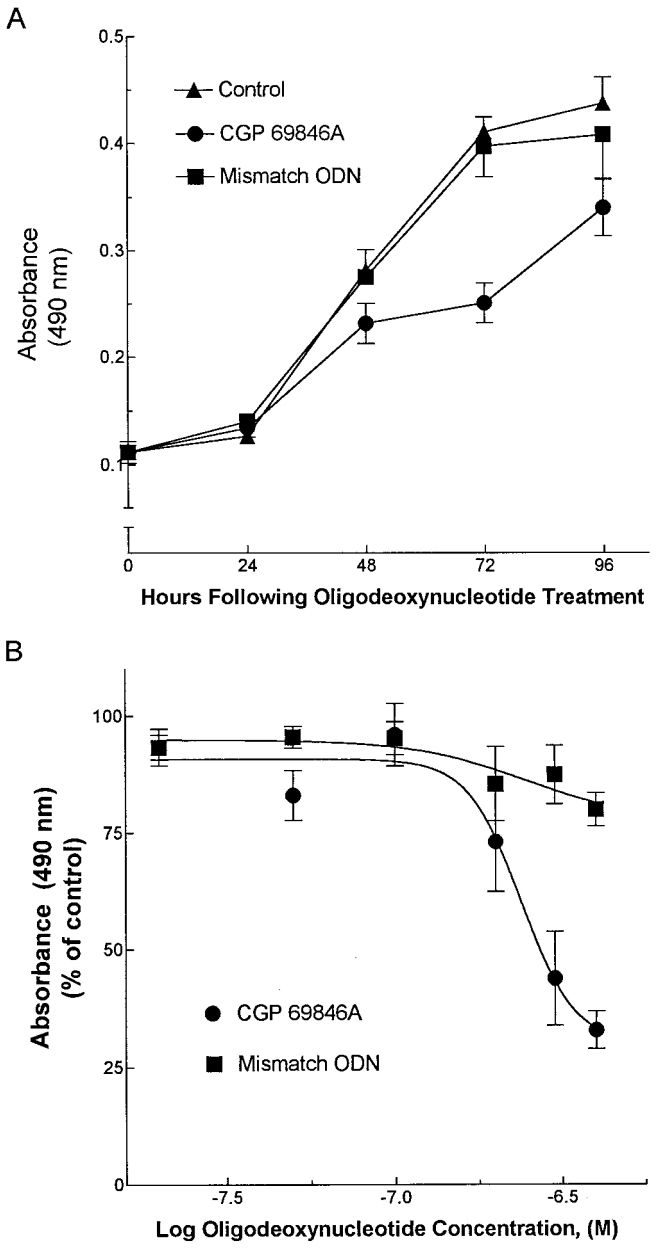

Fig. 2. Inhibition of serum-induced proliferation by CGP 69846A in human coronary artery smooth muscle cells. Growth-arrested cells were incubated with ODNs and subsequently stimulated with serum for the indicated time periods. A, Time course for the antiproliferative effects induced by CGP 69846A. Cells were exposed to 400 nM CGP 69846A or mismatched control ODN, and cellular proliferation was assessed by the MTS conversion assay. Values are mean ± standard error obtained from one of three similar experiments. B, Dose-response curve for inhibition of serum-induced proliferation by CGP 69846A. Cells were treated with increasing concentrations of ODN, and cell proliferation was assessed after 72 hr. Values are mean ± standard error obtained from three separate experiments and expressed relative to cells that were not treated with ODN.

mRNA expression levels recovered progressively such that the Raf-1 antisense treatment effect was undetectable 120 hr after compound exposure. Simultaneously, we assessed the Raf-1 protein levels after cell treatment with 400 nM CGP 69846A (Fig. 4B). We observed a slow onset of decreasing cellular Raf-1 protein levels after antisense treatment. Raf-1 protein levels dropped by 30% compared with untreated cells

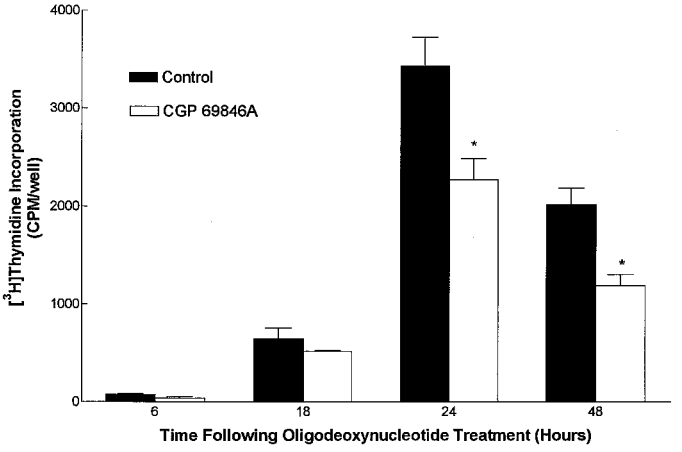

Fig. 3. Effects of CGP 69846A on serum-stimulated DNA synthesis in human coronary artery smooth muscle cells. Growth-arrested cells were incubated with 400 nM CGP 69846A or mismatched control ODN. Subsequently, cells were stimulated with serum for the indicated time periods and pulsed with [^{3}H]thymidine (5 mCi/ml) for the last 4 hr of this stimulation. The amount of [^{3}H]thymidine incorporated into the trichloroacetic acid-precipitable fraction was counted, and results are expressed as mean ± standard error obtained from one of two separate experiments. Basal [^{3}H]thymidine incorporation, which ranged from 200 to 400 dpm/well, was subtracted from each stimulated value.

24 hr after a single antisense treatment. At 48 and 72 hr, protein expression was reduced by ~60%. Subsequently, the Raf-1 protein levels recovered progressively from the antisense treatment such that 120 hr after treatment, the protein levels of Raf-1 were equal in the treatment and control samples. This study demonstrated the cell recovery from CGP 69846A exposure and therefore the reversibility of the pharmacodynamic effects. The time course pattern was unaltered regardless of whether the CGP 69846A antisense compound was added to proliferating smooth muscle cells or quiescent cells (data not shown).

We next investigated whether a second antisense cell treatment applied 48 hr from the first was able to further inhibit Raf-1 gene expression. Indeed, two applications of 400 nM CGP 69846A during that time interval further decreased Raf-1 protein levels to ~15% and ~20% of the control levels at the 72- and 96-hr time points, respectively (Fig. 4B). A second cell exposure to lipofectin by itself did not affect Raf-1 expression (data not shown).

Metabolic turnover rate of Raf-1 protein in human vascular cells. To assess the efficacy of CGP 69846A application in reducing Raf-1 gene expression, we compared the time-dependent decrease of cellular steady state levels of Raf-1 protein after antisense treatment with endogenous Raf-1 protein turnover rates in cycling cells. Human coronary artery smooth muscle cells were metabolically pulse-chase labeled with [^{35}S]methionine and [^{35}S]cysteine. Subsequently, anti-Raf-1 immunoprecipitates generated at four time points after cell labeling were analyzed by SDS-PAGE for the decomposition of radiolabeled Raf-1 protein (Fig. 5). An immunoprecipitation with an unrelated antiserum served as the negative control. The Raf-1 density values obtained from the autoradiogram at these time points were subjected to nonlinear regression, which yielded an approximate decay half-life of 30 hr for Raf-1 protein in our procedure. This metabolic decay rate for Raf-1 protein was thus comparable to the observed 85% decrease of Raf-1 gene expression within

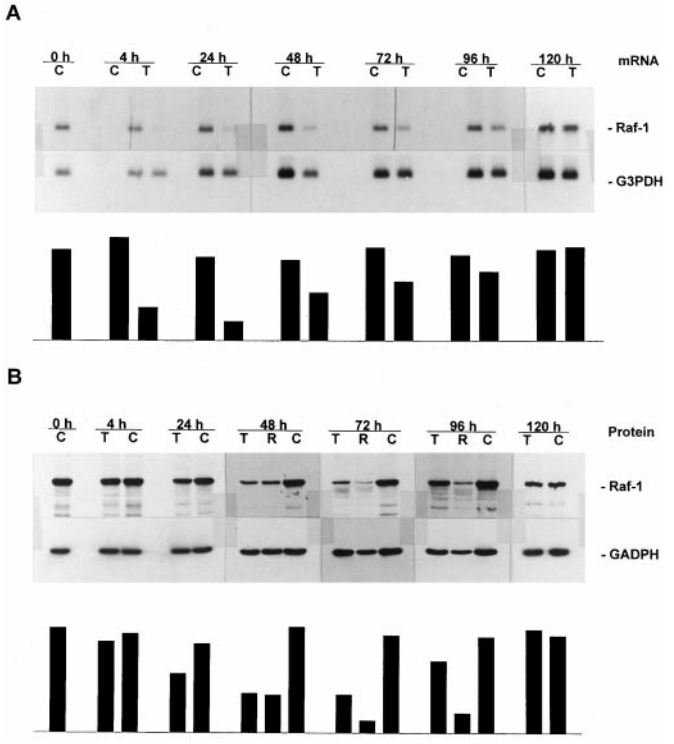

Fig. 4. Time course of Raf-1 mRNA and protein expression in human coronary artery smooth muscle cells after treatment with CGP 69846A and mismatched control ODN. Growth-arrested cells were treated with antisense ODNs at a concentration of 400 nM, re-exposed to growth stimulation, and analyzed for Raf-1 gene expression at the indicated time points. A, Raf-1 and G3PDH mRNA abundance was measured by Northern blotting and represented in normalized form (*bar graph*). Samples (*T*) were subjected to one antisense treatment. Control cell samples (*C*) were mock-treated. B, Raf-1 and GAPDH protein expressions were assessed by Western blotting and normalized Raf-1 protein levels (*bar graph*). Samples (*T*) were subjected to one antisense treatment. Samples (*R*) were subjected to an additional treatment period after 48 hr. Control cell samples (*C*) were mock-treated. One representative experiment of two is shown.

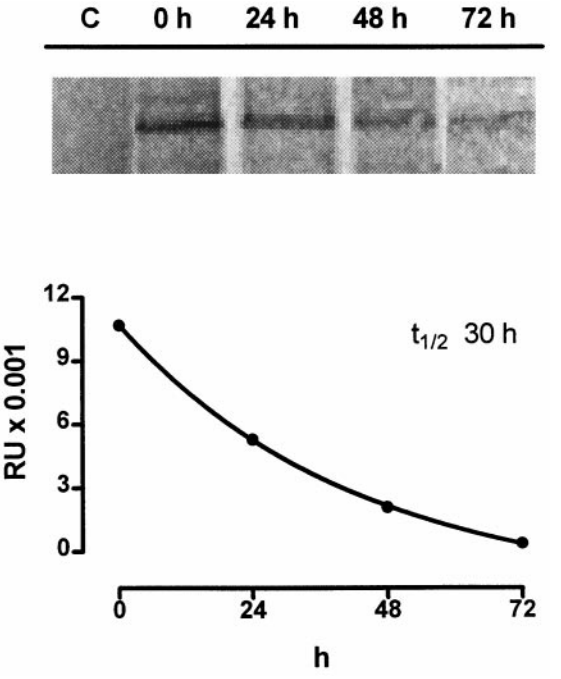

Fig. 5. Metabolic turnover rate of Raf-1 protein in human coronary artery smooth muscle cells. Cells were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine; subsequently, Raf-1 was immunoprecipitated at the indicated time points. Immunoprecipitates were analyzed by SDS-PAGE. The Raf-1 density values indicated as relative units (*RU*) were subjected to nonlinear regression analysis to calculate the decay half-time ($t_{½}$). The negative control lane (*C*) depicts an immunoprecipitation with an unrelated antiserum. One representative experiment of two is shown.

72 hr induced after two applications of 400 nM CGP 69846A to smooth muscle cells (Fig. 4B).

Discussion

We demonstrated that treatment of cultured human coronary artery smooth muscle cells with the antisense ODN CGP 69846A specifically reduced Raf-1 mRNA and protein expression as well as serum-induced cell proliferation. This compound was most potent in reducing Raf-1 mRNA and protein levels after 24 and 48 hr of cell exposure, respectively, with corresponding IC_{50} values of ~100 and ~300 nM. Proliferation ultimately was inhibited with an IC_{50} value of ~300 nM after 72 hr. These results reflect a serial time- and dose-dependent mechanism to achieve inhibition of cell cycle progression.

Activated Raf-1 kinase has been shown to stimulate cell cycle progression (Kolch *et al.*, 1991; Leevers *et al.*, 1994; Marshall, 1994). The essential role of the Ras-MAPK pathway in the transition from the quiescent to the DNA replication phase of the eukaryotic cell cycle has been demonstrated by the inhibition of MAPK, which blocked the induction of DNA synthesis by activated Ras (Sun *et al.*, 1994). In quiescent cells, inactivation of Ras prevented entry into the cell cycle, whereas in cycling cells, it caused an arrest in G1 phase (Peeper *et al.*, 1997). The observation that Raf-1 is activated during mitosis indicated that Raf-1 may also play a role in traversal of the G2/M phase of the cell cycle (Laird *et al.*, 1995; Lovric and Moelling, 1996). We show that down-regulation of Raf-1 gene expression by CGP 69846A reduced the rate of cell cycle progression in the absence of any detectable cytotoxic effects.

mRNA is a short-lived intermediary in the transfer of genetic information from DNA to protein. RNA decay rates determine the steady state level of a mRNA sequence and therefore its translation into an amino acid sequence. By using relative density values obtained from densitometric scanning of the autoradiographic Raf-1 mRNA and protein bands illustrated in Fig. 4, we generated rough estimations of antisense ODN half-lives and Raf-1 protein decrease rates. Phosphorothioate ODNs have been presumed to induce ribonuclease H-mediated mRNA cleavage on hybridization to their target transcript (Crooke *et al.*, 1995; Monia *et al.*, 1993; Wagner *et al.*, 1993). After antisense ODN application, cellular Raf-1 mRNA levels decreased rapidly within 24 hr to ~20% of the control level and recovered subsequently within 96 hr to the endogenous level. Accordingly, the recovery of decreased Raf-1 protein levels to endogenous levels after a single antisense ODN application occurred within ~120 hr (Fig. 4). Nonlinear regression analysis of the recovering Raf-1 mRNA density values (normalized to control) generated a mRNA doubling time of ~50 hr. This value of 50 hr can be interpreted as the half-life of active intracellular CGP 69846A in our cell culture system because the intrinsic

mRNA turnover rate is presumed to occur faster. The chemical half-life of an average mRNA species in mammalian cells was estimated to last only several hours (Tuite, 1996). The intracellular activity of phosphorothioate ODNs may be time-limited by their susceptibility to nucleolytic degradation or cellular excretion.

Exposure of smooth muscle cells to a single treatment of 400 nM CGP 69846A decreased Raf-1 protein levels within 48 hr to ~40% of the untreated control level. A second application of the antisense compound after 48 hr was necessary to maintain a reduced Raf-1 mRNA level and therefore further decrease Raf-1 protein levels. The repeated application of CGP 69846 decreased the cellular Raf-1 protein concentration over 72 hr to ~15% of the control concentration. Nonlinear regression analysis of the normalized densitometric Raf-1 protein values measured over these 72 hr generated a 50% decay rate of Raf-1 protein of ~33 hr.

To assess the efficacy of CGP 69846A in decreasing Raf-1 protein levels, we measured the endogenous turnover rate of Raf-1 in cycling smooth muscle cells. With the assumption that an antisense treatment entirely suppresses cellular protein synthesis, one would expect the antisense-induced decay rate to be equal to the endogenous protein turnover rate; therefore, cells were metabolically pulse-chase labeled and the decomposition of immunopurified Raf-1 protein was subsequently monitored over 72 hr. Nonlinear regression analysis of the densitometric Raf-1 values generated a 50% decay rate for endogenous Raf-1 protein of ~30 hr. The endogenous Raf-1 decay rate is therefore comparable to the antisense-induced protein decay rate, indicating that the efficiency of CGP 69846A in decreasing Raf-1 protein levels is rate-limited by the endogenous protein turnover rate. Indeed, the potency of CGP 69846A in reducing protein and proliferation (IC_{50} ~ 300 nM after 48 hr) was not predicted by its potency in reducing Raf-1 mRNA (IC_{50} ~ 100 nM after 24 hr). The pharmacodynamic effects of CGP 69846A antisense ODN are therefore limited by the duration of its intracellular activity rather than by its ability to transiently decrease mRNA levels because long-lived protein will not be significantly affected by transient decreases in mRNA.

Pharmacologically, to maintain a decreased steady state Raf-1 protein level *in vitro*, we suggest a repeated CGP 69846A application within the time range of the ODN and endogenous protein decay rates ($t_{½}$ = ~50 and ~30 hr, respectively). Maintaining decreased cellular steady state levels of Raf-1 gene expression by repeated ODN application or steady state exposure to ODNs might limit progression through the cell cycle over a prolonged period of time; however, it is not known whether repeated administration of antisense would further reduce cell proliferation. Residual Raf-1 protein or parallel growth signaling pathways may limit the effects of our treatment protocol on cell proliferation.

Cellular Raf-1 protein levels in quiescent or actively proliferating human coronary artery smooth muscle cells were equally expressed, indicating that serum starvation for 48 hr was insufficient to significantly down-regulate cellular Raf-1 protein. The application of CGP 69846A antisense ODNs to quiescent smooth muscle cells before subsequent growth stimulation as well as to actively proliferating cells did not alter the efficiencies of growth inhibition (data not shown); therefore, it is hypothesized that this ODN may also be used to prevent the transition of quiescent vascular smooth muscle cells into the pathologically hyperproliferating cells seen after percutaneous transluminal coronary angioplasty. Local delivery of CGP 69846A endoluminally after angioplasty may therefore prevent occurrence of vessel restenosis as well as circumvent problems of eventual systemic compound side effects.

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