Resveratrol causes cell cycle arrest, decreased collagen synthesis, and apoptosis in rat intestinal smooth muscle cells

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Garcia P, Schmiedlin-Ren P, Mathias JS, Tang H, Christman GM, Zimmermann EM. Resveratrol causes cell cycle arrest, decreased collagen synthesis, and apoptosis in rat intestinal smooth muscle cells. Am J Physiol Gastrointest Liver Physiol 302: G326–G335, 2012. First published November 3, 2011; doi:10.1152/ajpgi.00083.2011.—One of the most difficult and treatment-resistant complications of Crohn’s disease is the development of fibrotic intestinal strictures due to mesenchymal cell hyperplasia and collagen deposition. Resveratrol, a phytoalexin found in berries, peanuts, grapes, and red wine, has been shown to inhibit fibrosis in vasculature, heart, lung, kidney, liver, and esophagus in animal models. Resveratrol has also been shown to inhibit oxidation, inflammation, and cell proliferation and to decrease collagen synthesis in several cell types or animal models. The aim of this study was to determine whether resveratrol has antifibrotic effects on intestinal smooth muscle cells. Responses to resveratrol by cultured smooth muscle cells isolated from colons of untreated Lewis rats were examined; this rat strain is used in a model of Crohn’s disease with prominent intestinal fibrosis. A relative decrease in cell numbers following treatment with 50 and 100 μM resveratrol was evident at 24 h (P ≤ 0.005). This effect was largely due to cell cycle arrest, with an increase in the percent of cells in S phase from 8 to 25–35% (P < 0.05). Cell viability was unchanged until 2–3 days of treatment when there was a 1.2- to 5.0-fold increase in the percent of apoptotic cells, depending on the assay (P < 0.05). Expression of collagen type I protein was decreased following treatment with resveratrol for 24 h (to 44 and 25% of control levels with 50 and 100 μM resveratrol, respectively; P < 0.05). Expression of procollagen types I and III mRNA was also decreased with resveratrol treatment. Resveratrol (50 μM) diminished the proliferative response to TGF-β1 (P = 0.02) as well as IGF-I-stimulated collagen production (P = 0.02). Thus resveratrol decreases intestinal smooth muscle cell numbers through its effects on cell cycle arrest and apoptosis and also decreases collagen synthesis by the cells. These effects could be useful in preventing the smooth muscle cell hyperplasia and collagen deposition that characterize stricture formation in Crohn’s disease.

Crohn’s disease; inflammatory bowel disease; fibrosis; stricture

FIBROSIS IN Crohn’s disease (CD) is a consequence of chronic transmural inflammation causing hyperplasia of cells with a smooth muscle cell phenotype and dysregulated turnover of collagen and other extracellular matrix (ECM) components (reviewed in Ref. 7). Fibrosis in CD has devastating clinical consequences including the development of intestinal strictures and fistulae that cause significant patient morbidity and often result in the need for surgical intervention. Currently no therapeutic agents have been shown to specifically decrease fibrosis in CD. Furthermore, it is unclear whether potent immuno-suppressive agents, which are the mainstay of therapy for serious CD, decrease tissue fibrosis. Agents that prevent or slow the proliferation of smooth muscle cells and decrease excessive collagen deposition may provide new therapeutic avenues or, more likely, be used as an adjunct to current immunomodulatory therapy of fibrostenosing CD.

Although the full molecular mechanisms of stricture formation are not known, several factors, including IGF-I, TGF-β1, connective tissue growth factor, platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor, have been reported to both increase cellular proliferation (intestinal smooth muscle cells or myofibroblasts) and increase synthesis of ECM or collagen (7, 22, 41). The proinflammatory cytokines TNF-α and IL-1β, which both regulate and are regulated by NF-κB (reviewed in Ref. 2), are key mediators of the intestinal inflammation in CD and have been reported to increase proliferation of intestinal smooth muscle cells in vitro (30).

Resveratrol (trans-3,5,4’-trihydroxystilbene) is a natural phytoalexin found in berries, peanuts, grapes, and red wine. Resveratrol has been shown to reduce inflammation and mucosal damage in two rat models of colitis (23, 28) and in experimental caustic esophageal burns (39). Relevant to fibrosis and strictures, resveratrol has been shown to inhibit proliferation (32), induce apoptosis (32), and cause cell cycle arrest (29, 32) in cultured smooth muscle cells, as well as to inhibit intimal hyperplasia in an in vivo model of vascular injury (43). Resveratrol has also been shown to inhibit fibrosis in liver (9, 16, 24), kidney (25), heart (37), and vasculature (18) in rat models of disease and to inhibit stricture formation in experimental caustic esophageal burns (39).

On the molecular level, resveratrol has been shown to reduce activation of NF-κB in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis model (28) and in the streptozotocin diabetic rat model of vascular disease (18), as well as in several types of cancer cells (3, 11, 36). Resveratrol reduced collagen I overexpression in the hearts of rats in a model of metabolic syndrome (37), reduced collagen deposition and cross linking in the wall of the aorta in the streptozotocin diabetic rat model (18), and reduced collagen types I and III expression in cultured leiomyoma cells (8). Resveratrol reduced the mRNA expression of the inflammatory mediators TNF-α and IL-1β, as well as the mRNA expression of collagen type I and TGF-β in the dimethylsulfatoamine model of liver fibrosis in rats (16, 24). In the unilateral ureteral obstruction mouse model of interstitial renal fibrosis, the reduction by resveratrol of the extent of both the macrophage infiltration and the fibrotic response was accompanied by marked reduction of Smad3 acetylation (25),
which is an important mechanism by which TGF-β1 stimulates the fibrotic process (reviewed in Ref. 12).

Resveratrol has been shown to have antiproliferative effects in a variety of cell types through induction of cell cycle arrest and/or apoptosis. Specifically, resveratrol has been shown to cause G1- or S-phase arrest in several cancer cell lines (3, 4, 11, 20, 35, 36, 40), leiomyoma cells (8), and the Rat-1 normal rat fibroblast cell line (35) and to induce apoptosis in cancer cells (4, 11, 20) and leiomyoma cells (8).

In CD, cells with a smooth muscle cell phenotype, including smooth muscle cells and myofibroblasts, are the primary cells responsible for stricture formation. Although previous studies have shown that resveratrol decreases vascular smooth muscle cell proliferation via cell cycle arrest and apoptosis (29, 32), there have been no studies on the effects of resveratrol on intestinal smooth muscle cells. The aim of this study was to determine whether resveratrol decreases proliferation of and collagen synthesis by intestinal smooth muscle cells. In addition, the effects of resveratrol on cell cycle arrest and apoptosis were studied to determine a mechanism by which an observed relative reduction in cell numbers could have been mediated.

MATERIALS AND METHODS

Animals. The use of animals in these studies was approved by the University Committee on Use and Care of Animals of the University of Michigan, and their guidelines were adhered to. Female specific pathogen-free (SPF) Lewis rats weighing 125–140 g (Charles River Laboratories, Wilmington, MA) were housed under SPF conditions.

Cell culture. The colon was removed from each of four euthanized rats. Rat intestinal smooth muscle cells (RISM) were isolated from the muscularis externa of the colons by a standard collagenase digestion as described previously (13, 42). RISM were cultured in DMEM containing 25 mM HEPES (GIBCO Invitrogen, Carlsbad, CA) and supplemented with 15% FBS (HyClone, Logan, UT), 0.1 mM non-essential amino acids, 100 U/ml penicillin G, and 100 U/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in room air. Medium was changed every 2–3 days. Cells cultured in this manner have a morphology characteristic of smooth muscle cell and express smooth muscle α-actin, a marker of smooth muscle cell differentiation, at least through passage 20 (unpublished observations, E. M. Zimmermann). Several passages by standard trypsinization were necessary to obtain adequate numbers of cells for experimentation. Cells from passages 7–12 were used for these studies. Cells from the Lewis strain of rat were used because this strain is used in one of the only well-characterized animal models of CD with prominent intestinal fibrosis (33, 41). This model is used for studying mechanisms of fibrosis and for testing anti-inflammatory and antiangiogenic drugs.

Resveratrol treatment. A 100 mM stock solution was prepared by dissolving resveratrol (Sigma Chemical, St. Louis, MO) in sterile cell culture-grade DMSO (Sigma). Working dilutions were made in culture medium immediately before use, while maintaining a uniform 0.1% concentration of DMSO. Control cultures were exposed to 0.1% DMSO in medium but no cells. Resveratrol and its stock solution were stored at −20°C, protected from light. For the experiments, RISM were seeded at 0.8–1.7 × 104 cells/cm2 in multiwell plates. Treatments were started when the cells were 60–80% confluent. The day on which treatment was started was considered day 0.

Cytokine treatment. In some cases, RISM were transitioned to serum-free medium. Twenty-four hours later, treatment with TGF-β1 (1 ng/ml; recombinant human; R&D Systems, Minneapolis, MN) or IGF-1 (100 ng/ml; recombinant rat; R&D) in serum-free medium, with or without resveratrol (50 μM), was begun. The medium was changed daily over the course of the 1- to 3-day treatment period.

Assessment of proliferation. Triplicate wells of RISM grown in 24-well plates were treated with each concentration of resveratrol for each of three durations of treatment. At the end of incubation, the cells were removed from the wells by trypsinization. Cells were counted in a hemacytometer and assessed for viability based on their ability to exclude Trypan blue. The total number of cells in each well was calculated, and results were expressed relative to those of untreated cells trypsinized on day 0 (Fig. 1A) or relative to vehicle-exposed cells trypsinized at the same time point (Fig. 1C). This experiment was done twice.

Assessment of viability by MDH assay. Mitochondrial dehydrogenase (MDH) activity was assayed by using the colorimetric Cell Counting Kit-8 from Dojindo Molecular Technologies (Gaithersburg, MD) according to the manufacturer’s instructions. Concurrent with the cultures for cell counting described above, RISM were seeded into 96-well plates for the MDH assay. Duplicate wells were treated with each concentration of resveratrol for each of three durations of treatment. At the end of incubation, 10 μl of CCK-8 reagent was added to the 100 μl of medium in each of the appropriate wells, including some wells containing medium but no cells. The plates were returned to the 37°C incubator for 2 h; then absorbance at 450 nm, with reference wavelength 655 nm, was measured in a plate reader. Readings from unseeded wells were used for subtraction of background. Results were expressed relative to those of vehicle-exposed cells assayed at the same time point. This experiment was done twice.

Cell cycle analysis. Duplicate or triplicate wells of RISM grown in six-well plates were treated with varying concentrations of resveratrol for 24 h. At the end of treatment, cells were trypsinized and then resuspended in culture medium. A portion of each cell suspension was stained with Trypan blue and counted in a hemacytometer. The remaining cells were resuspended in cold PBS to a concentration of 2 × 106 cells/ml. Cells were fixed for 20 min on ice after the dropwise addition of an equal volume of cold absolute ethanol. The fixed cells were pelleted by centrifugation, then resuspended in one volume of PBS. An equal volume of 50 μg/ml propidium iodide (PI; Sigma) with 100 μg/ml RNase Type I (Roche Applied Science, Indianapolis, IN) in PBS was added to each tube. After incubating for 20 min at room temperature in the dark, the cells were analyzed by flow cytometry by use of a BD Biosciences FACSCalibur Cytometer. Cell cycle analysis was done by using ModFitLT V3.1 (FlowJo) software. This experiment was done four times.

Caspase-3/7 activation assay. Caspase-3/7 activity was measured via the luminescence Caspase-Glo 3/7 assay from Promega (Madison, WI) according to the manufacturer’s instructions. Duplicate wells of RISM grown in 96-well plates were treated with varying concentrations of resveratrol. Reagent (100 μl) was added to the 100 μl of medium present in each well, including some wells containing medium but no cells. After 1 h incubation at room temperature in the dark, the plate was read in a luminescence plate reader (Wallac 1420 Victor 3 Multilabel Counter, PerkinElmer, Waltham, MA). Readings from unseeded wells were used for subtraction of background. Cells treated with 1 μM staurosporine (Sigma) for 24 h served as positive controls and had readings greater than or equal to fourfold those of vehicle-exposed cells or relative to vehicle-exposed cells.

Annexin V-FITC staining for flow cytometry and for microscopy. The apoptotic event of exposure of phosphatidylserine on the outer surface of the plasma membrane was assessed by using the Annexin V-FITC Apoptosis Detection Kit I from BD Biosciences (San Jose, CA) according to the manufacturer’s instructions. Duplicate wells of RISM grown in six-well plates were treated for 3 days with varying concentrations of resveratrol. At the end of treatment, the culture supernatants were collected to capture any nonadherent cells. Resveratrol concentration and duration of treatment was represented in at least four experiments.
Lab-Tek; Thermo Fisher Scientific, Rochester, NY). Cells were
PI only. Events in the lower right quadrant (FITC
cells, cells stained with annexin V-FITC only, and cells stained with
immediately analyzed by flow cytometry. Controls included unstained
cells, cells stained with annexin V-FITC only, and cells stained with
PI only. Events in the lower right quadrant (FITC+, PI−) were judged to
represent apoptotic cells. This experiment was done twice.

For microscopy, the cells were seeded onto chamber slides (Nunc Lab-Tek; Thermo Fisher Scientific, Rochester, NY). Cells were
treated for 2 days with varying concentrations of resveratrol. At the end of treatment, the cells were stained for 15 min at room temper-
ature in the dark with use of the reagents from the kit above. A
coverslip was applied with Prolong Gold anti-fade mounting medium
(Molecular Probes; Invitrogen, Carlsbad, CA), and the cells were
examined via an epifluorescence microscope with digital camera
attached.

* TUNEL staining for flow cytometry. The late apoptotic event of
DNA cleavage was assessed by using the APO-BRDU Kit from
Phoenix Flow Systems (San Diego, CA). Fixation, permeabilization,
and staining were done according to the kit instructions. Quantitation
of apoptotic cells was then done by flow cytometry. After aspirating
the medium from RISM grown in six-well plates and washing the
twice to remove any nonadherent cells, 2-day treatment with
varying concentrations of resveratrol was begun. At the end of
treatment, the culture supernatants were collected to capture any
nonadherent cells. Adherent cells were released from the wells by
trypsinization and added to the tubes containing the culture superna-
tants. A portion of each resulting cell suspension was stained with
Trypan blue and counted in a hemacytometer. Remaining cells were fixed,
permeabilized, and labeled with bromodeoxyuridine (BrdU).
Incorporated BrdU was detected by incubation with the FITC-labeled
antibody provided, followed by incubation with RNase A/PI solution
and analysis by flow cytometry. Controls included unstained cells,
cells stained with BrdU-FITC only, and cells stained with PI only.
Events in the right upper quadrant (FITC+, PI+) were judged to
represent apoptotic cells. Cells treated with 1 μM staurosporine (Sigma)
for 24 h served as positive controls and had 65–75% of cells in the
upper right quadrant, which was ~25-fold the value for
vehicle-exposed cells. Each concentration of resveratrol was repre-
sented by two to four wells in at least one of two experiments.

Western immunoblot analysis. Duplicate wells of RISM grown in
six-well plates were treated for 24 h with varying concentrations of
resveratrol. At the end of treatment, the medium was aspirated and the
wells were washed three times with 2 ml of cold PBS. Lysis buffer
[0.5% Triton X-100, 2 mM EDTA, 2 mM EGTA, 150 mM sodium
chloride, 25 mM Tris·HCl (pH 7.4), 10% glycerol] with protease
inhibitors (1 mM PMSF, 0.2 TIIu/ml aprotinin, 10 μg/ml leupeptin; all
from Sigma) was added to each well (100 μl/well). The cells were
scraped from the wells and transferred to microcentrifuge tubes,
which were placed on ice for 30 min, then centrifuged at 3,000
g for 10 min at 4°C and then at 10,000 g for 2 min. Supernatants were
transferred to fresh tubes and stored at −80°C pending analysis.

Protein concentrations of the lysates were determined by Bradford
protein assay (6) using bovine serum albumin standards. The lysates
(8 μg protein/lane) were subjected to SDS-PAGE on 8% nonreducing
gels. The size-separated proteins were electrophoretically transferred
to polyvinylidene difluoride membranes (Amersham Hybond-P, GE
Healthcare Bio-Sciences, Piscataway, NJ). The membranes were
blocked with 5% nonfat dry milk in TBS-T [20 mM Tris (pH 7.5), 150
mM NaCl, 0.3% Tween 20] for 1 h, then incubated with 1:1,000 rabbit
antibody against collagen type I (Rockland, Gilbertsville, PA) overnight at 4°C.
After washing, the blots were incubated with 1:10,000 horseradish
peroxidase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology,
Santa Cruz, CA) for 1 h at room temperature. The blots were again
washed, then incubated with ECL Western Blotting Detection Re-
agents (Amersham). Exposures with Hyperfilm ECL (Amersham)
were done. Densitometry of digital scans of the developed films was
done using National Institutes of Health (NIH) Image software (avail-
able online at http://rsb.info.nih.gov/ihimage/). Duplicate blots were
done for each of two experiments.

Real-time quantitative PCR. RISM cultures grown in six-well
plates and treated concurrently with those used for immunoblot
analysis were used, with duplicate wells in each of two experiments.
Total RNA was isolated using the Qiagen RNAeasy Mini Kit (Qiagen-
USA, Valencia, CA; cell lysis in 350 μl/well Buffer RLT with added
2-mercaptoethanol; single 30 μl elution of RNA from column).
Reverse transcription of a 0.5 μg portion of each RNA sample was done by using TaqMan RT Reagents (Applied Biosystems, Foster City, CA) in a total volume of 25 μl. PCR reaction mixtures consisted of 2 μl of cDNA and 18 μl of TaqMan Gene Expression Master Mix (Applied Biosystems) to which primers and probe had been added. Real-time PCR was done by using an ABI PRISM 7300 instrument (Applied Biosystems) with PCR reaction conditions of 2 min at 50°C, 10 min at 95°C, and then 40 cycles, each consisting of 15 s at 95°C and 1 min at 60°C.

Primer-probe combinations for rat procollagen types I and III were designed to span an intron and were obtained from Applied Biosystems. (Rat procollagen type I: Forward Primer 5’ TTCACCTACAGCACCCTTGTG 3’; Probe 5’ TGACAGATCCACCCGAACCTTGG 3’; Backward Primer 5’ TTGGTGTTTTTGAATTCTCGACT 3’. Rat procollagen type III: Forward Primer 5’ GGAATCTGTGAAATCATGTCCAACCT 3’; Probe 5’ TGCCAGATATTATCTCCCAATTCGAC 3’; Backward Primer 5’ CATTCTCTCGACTCAGACTTG 3’.) A TaqMan Gene Expression Assay for GAPDH was run concurrently, and the procollagen mRNA expression relative to that of GAPDH was determined.

Statistical analysis. Experimental results were assessed for significance by one-tailed, unpaired t-tests. A P value less than 0.05 was considered significant.

RESULTS

Effect of resveratrol on proliferation and cell numbers. To determine the effect of resveratrol on RISM proliferation, populations of cultured cells were exposed to varying concentrations of resveratrol, and changes in cell number were monitored at 1, 3, and 5 days (Fig. 1A). As expected, there was some decline in the rate of increase in cell numbers over time as the cells approached confluence. However, relative to cell numbers at day 0, the populations of cells exposed to 0, 1, or 10 μM resveratrol continued to double approximately every 2 days, on average; cells treated with 50 μM resveratrol doubled every three days. Cell numbers in cultures treated with 100 μM resveratrol for 1, 3, or 5 days were unchanged from pretreatment values.

When cell numbers in resveratrol-treated cultures were compared with those of vehicle-exposed cells at the same time point, differences were significant at days 1, 3, and 5 of treatment for both 50 and 100 μM resveratrol, and the intensity of the effect was dose dependent. Cell numbers in cultures treated with 50 μM resveratrol were ~0.4- to 0.5-fold control values (P ≤ 0.005), whereas those treated with 100 μM resveratrol were ~0.4-, 0.2-, and 0.15-fold control values at days 1, 3, and 5, respectively (P < 0.002).

Effect of resveratrol on cell viability. Viability of the trypsinized cells, as assessed by Trypan blue exclusion, remained 94–100% regardless of treatment or time point. In addition to using Trypan blue exclusion, viability was also assessed by measuring MDH activity in parallel cultures (Fig. 1B). A decrease in MDH activity was seen in wells treated with 50 μM resveratrol for 5 days (P = 0.000007) or 100 μM resveratrol at all three time points (P = 0.03, 0.006, and 0.00002 for days 1, 3, and 5, respectively). However, because the MDH activity assay is performed by adding the substrate directly to the culture wells, the results will be affected by the numbers of cells present. Therefore, to better interpret the MDH assay results, MDH activity was plotted against the cell number data from the parallel cultures, expressing both variables as fold the vehicle control value at the same time point. There was a linear relationship consistent with viability rates among the variably treated cultures (Fig. 1C).

However, the data point (marked with an arrow) representing cultures treated with 100 μM resveratrol for 5 days did not fit the curve, indicating that at least some of these cells no longer contained the expected levels of active MDH. Although, as noted above, these cells continued to be able to exclude Trypan blue, the diminished MDH activity suggests that an increased proportion of the cells were approaching cell death. As noted above and as more readily apparent in Fig. 1A where cell numbers are expressed relative to day 0, the absolute cell numbers were the same at all three time points with 100 μM resveratrol treatment. Therefore, the observation that the linear relationship between MDH activity and cell numbers applies at days 1 and 3 but not at day 5 of 100 μM resveratrol treatment cannot be explained in terms of loss of linearity of the assay.

Effect of resveratrol on cell cycle. To further elucidate the mechanism by which treatment with resveratrol resulted in a relative decrease in cell numbers, the effect of resveratrol on cell cycle was examined. The percentages of cells in phases G1, S, and G2 of the cell cycle were determined in trypsinized cells from RISM cultures treated with varying concentrations of resveratrol for 24 h. Figure 2 shows flow cytometry tracings from a representative experiment. Arrest in S phase was seen following treatment with 50 or 100 μM resveratrol, whereas no effect was seen for concentrations ≤10 μM. The combined results of four such experiments are shown in Table 1. The percentage of cells in S phase increased from 8 to 28% of gated cells with 50 μM resveratrol (P = 0.01) and to 35% with 100 μM resveratrol (P = 0.004). A concomitant decrease in the percentage of cells in G1 phase was seen.

Effect of resveratrol on apoptosis. The finding above that the reduction of MDH activity in cells treated with 100 μM resveratrol for 5 days could not be fully explained by the effect of resveratrol on cell cycle arrest, together with multiple reports in the literature that resveratrol induces apoptosis in
Cultured rat intestinal smooth muscle cells (RISM) were treated for 24 h with resveratrol (Res) at the indicated concentrations (brackets denote concentration). Trypsinized cells were incubated with DNase-free RNase, stained with propidium iodide, and analyzed by flow cytometry. Peak integration was done by use of ModFitLT V3.1 (PMac) software. Values are means and SD of the results from 4 separate experiments, each with duplicate or triplicate cultures. *P < 0.05; †P < 0.005; ‡P < 0.0005.

other cell systems, prompted evaluation of apoptosis as a possible mechanism contributing to the relative decrease in cell numbers seen in resveratrol-treated RISM cultures. The extrinsic and intrinsic pathways of apoptosis have in common the activation of effector caspases 3, 6, and 7. These effector caspases drive many of the cellular changes characteristic of apoptosis such as exposure of phosphatidylserine on the external surface of the plasma membrane and DNA fragmentation.

Caspase-3/7 activation was measured by a luminescent assay (Caspace-Glo 3/7 assay, Promega) that involves lysis of the cells and incubation with a substrate from which activated caspase-3 and/or -7 release a luminescent product. Surprisingly, a decrease in caspase activation was seen after 1 day of treatment with 75 and 100 μM resveratrol compared with vehicle-exposed cells (P < 0.0005; Fig. 3A). This finding was also seen with 2 and 3 days of treatment with these higher concentrations of resveratrol (75 μM, P < 0.05; 100 μM, P = 0.005). In contrast, there was no difference in caspase activity in cells treated with 10, 25, and 50 μM resveratrol for 1 day compared with vehicle-exposed cells. However, with 2 or 3 days of treatment with these lower concentrations of resveratrol there was increased caspase-3/7 activation (2 days, P < 0.0005; 3 days, P < 0.05).

Because the cells were lysed directly in the culture wells for this assay, differences in cell numbers due to cell cycle arrest could at least partially explain the observed decrease in caspase activation observed with the higher concentrations of resveratrol. In an effort to correct for this, a ratio of luminescence to estimated cell count was calculated and plotted against the concentration of resveratrol used to treat the cells (Fig. 3B). This was done using mean luminescence data from 3-day treated cultures available from 5–7 of the experiments shown in Fig. 3A (expressed as fold vehicle control) and mean cell count data from 3-day treated cultures for which representative data are shown in Fig. 1A (but expressed for the purpose of this ratio as fold vehicle control). The plot suggests increased rather than decreased caspase-3/7 activation in the cells treated with both 50 and 100 μM resveratrol.

Following caspase activation, several cellular changes occur in the process of apoptosis. The externalization of phosphatidylserine marks apoptotic cells for phagocytosis and can be measured by flow cytometry following trypsination and then incubation with FITC-labeled annexin V. Cells are also stained with PI, which is excluded by intact cellular membranes and helps to distinguish apoptotic from necrotic cells. RISM cultures were treated with 50 or 100 μM resveratrol for 3 days, trypsinized, stained as described, and then analyzed by flow cytometry. Cells that stained with FITC-annexin V and excluded PI were identified as apoptotic (lower right quadrant; Fig. 4A). By this method, 3-day treatment with 50 and 100 μM resveratrol resulted in an increase in the proportion of apoptotic cells [2.40-fold (P = 0.03) and 5.2-fold (P = 0.00004), respectively; Fig. 4B]. Because of concerns that the process of trypsination might cause exposure of phosphatidylserine, fluorescence microscopy was used to examine cells incubated with FITC-annexin V and PI without trypsination (Fig. 4C). After incubation for 2 days with 100 μM resveratrol, increased numbers of cells clearly demonstrated staining with FITC-annexin V whereas very few stained with PI, consistent with apoptosis. Cells incubated with 0.5 μM staurosporine for 6.5 h were used as positive controls for apoptosis, with most of the cells staining with FITC-annexin V.

The fragmentation of DNA by endogenous endonucleases also occurs in apoptotic cells and can be detected with flow cytometric analysis of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-stained tryptsinized cells. Cells that stained with both FITC-BrdU and PI (used to confirm adequate permeabilization of membranes)
were identified as apoptotic (upper right quadrant, Fig. 5A). In cells treated with 75 and 100 μM resveratrol for 2 days, an ~2.2-fold increase in BrdU-positive cells was seen (P < 0.05, Fig. 5B). This represents an increase in BrdU-positive cells from 2.8% in vehicle-exposed cells to 6.9 and 5.8% in 75 and 100 μM resveratrol-treated cells, respectively. In contrast, staurosporine-treated control cultures (1 μM, 24 h) had 70% of gated cells BrdU positive (25-fold increase).

Effect of resveratrol on collagen expression. To examine the effects of resveratrol on collagen expression, levels of collagen type I protein were assessed by Western immunoblot, and effects of resveratrol on collagen expression, levels of collagen type I mRNA level to 72% (P = 0.02) and 60% (P = 0.003) of control values, respectively (Fig. 6C). After 2- or 3-day treatment with 50 or 100 μM resveratrol, there was a decrease in procollagen type I mRNA level to 72% (P = 0.02) and 60% (P = 0.003) of control values, respectively (Fig. 6C).

DISCUSSION

Resveratrol is currently being evaluated in clinical trials as a potential cardiovascular protective and colon cancer chemopreventive agent. Resveratrol has previously been shown to have anti-inflammatory, antiproliferative, and/or antifibrotic effects in both cell culture and animal models of colitis, cancer, cirrhosis, kidney disease, and atherosclerosis (4, 9, 16–18, 23–26, 28, 29, 32, 34, 37, 38, 43) and in cultured leiomyoma cells (8). These effects make resveratrol an attractive potential therapeutic agent for CD, a chronic inflammatory intestinal disease that can be complicated by intestinal strictures and colon cancer. In this study we investigated the effects of resveratrol on cell proliferation and collagen synthesis as well as the mechanism by which resveratrol decreases proliferation. We showed that resveratrol decreases RISM cell numbers through its effects on cell cycle arrest and apoptosis and also decreases collagen synthesis by the cells. In addition, we showed that resveratrol is able to diminish the stimulatory effects of TGF-β1 and IGF-I on RISM proliferation and collagen type I production, respectively. These results support the study of resveratrol for stricture prevention and, perhaps, stricture therapy in animal models of CD and eventually in patients with the disease.

Resveratrol caused a dose-dependent antiproliferative effect on RISM, consistent with a broad body of literature demonstrating resveratrol-induced growth inhibition across many cell types. More specifically, this result is consistent with data showing that resveratrol decreases vascular smooth muscle cell hyperplasia in models of atherosclerosis through cell cycle arrest and apoptosis (29, 32, 43). Minjoyan and Fujise (29) found that, in proliferating human vascular smooth muscle cells, low concentrations (6.25–12.5 μM) of resveratrol for 24 h caused cell cycle arrest but not apoptosis, whereas a higher concentration (25 μM) of resveratrol caused an increase in apoptosis in addition to cell cycle arrest. In those studies of
apoptosis, a 24-h pretreatment with resveratrol was given prior to growth stimulation of the cells by the addition of serum to the medium. Using bovine vascular smooth muscle cells, Poussier et al. (32) found that decreased proliferation and increased apoptosis occurred after 24-h treatment with 10 or 100 μM resveratrol in a dose dependent manner. The dose of resveratrol reported to be required to cause 50% growth inhibition (IC50) in cultured cells was 90 μM for normal rat fibroblasts (Rat-1 cell line; Ref. 35) and 20–150 μM for various cancer cell lines (20, 35). In the present study, the IC50 for growth inhibition of RISM after 24 h resveratrol treatment was ~100 μM (Fig. 1); S-phase arrest was demonstrated with 24-h exposure to moderate to high concentrations (50–100 μM) of resveratrol (Fig. 2, Table 1). Evidence of apoptosis was seen after 2–3 days of 50–100 μM resveratrol treatment of RISM (Figs. 3–5). These data are consistent with published work in other cell culture systems. Our data suggest that the principal effect of resveratrol on RISM is that of cell cycle arrest, with the effect on apoptosis being relatively minor.

We also showed a decrease in collagen type I protein expression after 24-h treatment of RISM with 10, 50, or 100 μM resveratrol (Fig. 6, A and B). The effect of resveratrol on collagen synthesis appears to be independent of effects on cell cycle progression and apoptosis since these effects were not detected at the 10 μM concentration. Unexpectedly, although resveratrol also decreased procollagen type I mRNA expression (Fig. 6C), this was not observed at the 10 μM concentration, was observed at day 2 but not days 1 or 3 at the 50 μM concentration, and was observed readily at the 100 μM concentration, but only at days 2 and 3. This may indicate that collagen type I protein expression is not regulated solely at the transcriptional level or may reflect differences in robustness of the assays. Procollagen type III mRNA expression was decreased in RISM treated for 2 or 3 days with 50 and 100 μM resveratrol (Fig. 6D). These findings are consistent with reports in the literature of decreased collagen types I and III protein or mRNA expression in rat models of organ fibrosis (16, 18, 24, 37) and in cultured leiomyoma cells (8).

Further investigation of the mechanisms responsible for the effects of resveratrol reported here are needed. Resveratrol has been shown to reduce activation of NF-κB (3, 11, 15, 18, 28, 36), which could play a role in the observed proapoptotic effect. Resveratrol treatment has also been found to reduce acetylation of Smad3 (25), which is an important mechanism by which TGF-β1 stimulates the fibrotic process (reviewed in Ref. 12). The interplay of the various complex pathways affected by resveratrol requires detailed examination beyond the scope of the present study.

An important consideration in evaluating the therapeutic potential of resveratrol is whether effective concentrations can be attained in the blood or target tissue. Human pharmacokinetic studies have shown that achievable blood levels of unconjugated resveratrol are in the range of 0.02–0.5 μM when doses of 0.6–5.0 g per day are given (1, 5) and that
conjugated forms are present in plasma at much greater concentrations than the parent compound (5). Others have reported increases of total plasma resveratrol levels (parent plus conjugates) of 0.6–1.0 μM following wine intake (31). Although these plasma levels are considerably lower than the 10–100 μM concentrations needed in our culture studies to cause cell cycle arrest, apoptosis, and decreased collagen synthesis in RISM, it is possible that local effects of orally administered resveratrol on the intact gut could occur without accompanying high plasma levels of resveratrol. In this regard and relevant to CD, enterohepatic circulation of resveratrol has been demonstrated experimentally in rats (27), increasing exposure of the gut to resveratrol. The ability to attain therapeutic levels of resveratrol in vivo is supported by whole animal studies in rats that have shown reduced inflammation and tissue damage in the DSS (dextran sodium sulfate) and TNBS models of inflammatory bowel disease (23, 28); chemopreventive effects of resveratrol in models of colon, esophageal, and breast cancer (3, 26, 34, 38); and decreased fibrosis in disease and injury models involving esophagus, liver, kidney, lung, heart, and vasculature (9, 16, 18, 21, 24, 25, 37, 39, 43). With regard to the high rates of resveratrol conjugation that have been reported to occur in animals and humans, to our knowledge,
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biological activity of the conjugates has not yet been ruled out. It has also been reported that flavonoids such as quercitin inhibit the conjugation of resveratrol and thereby increase resveratrol bioavailability (10), suggesting that dietary manipulations could perhaps allow therapeutic blood and tissue levels of resveratrol to be achieved. Thus further investigation of resveratrol as an antifibrotic agent for use in CD is warranted.

In CD, intestinal cells with a smooth muscle cell phenotype including resident smooth muscle cells and myofibroblasts are the primary agents of stricture formation. In the present study, resveratrol was shown to inhibit proliferation through cell cycle arrest, increase apoptosis, and decrease collagen synthesis in cultured rat intestinal smooth muscle cells. The data shown here are the first to demonstrate the antiproliferative and antifibrotic effects of resveratrol on intestinal smooth muscle cells. Further studies including in vivo testing in animal models of CD are needed to clarify mechanism, pharmacokinetics, and in vivo applicability of resveratrol for prevention or treatment of intestinal strictures.

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DISCLOSURES

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