

## Pravastatin Inhibits the Rho/CCN2/Extracellular Matrix Cascade in Human Fibrosis Explants and Improves Radiation-Induced Intestinal Fibrosis in Rats

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**Abstract** **Purposes:** Intestinal complications after radiotherapy are caused by transmural fibrosis and impair the quality of life of cancer survivors. Radiation fibrosis was considered permanent and irreversible, but recently, its dynamic nature was shown, providing new opportunities for the development of antifibrotic therapies. Among these new targets, we identified the Rho/ROCK pathway and thought to investigate whether pravastatin treatment inhibits Rho pathway activation and elicits an antifibrotic action.

**Experimental Design:** Rho and ROCK activities were monitored in human explants presenting radiation fibrosis remodeling after incubation with pravastatin. Subsequent modulation of CCN2, type I collagen, and fibronectin expression were assessed *ex vivo* and in intestinal smooth muscle cells derived from radiation enteropathy. Then, the therapeutic relevance of the antifibrotic action of pravastatin was explored *in vivo* in a rat model of chronic radiation fibrosis (19 Gy X-rays) treated with 30 mg/kg/d pravastatin in the drinking water.

**Results:** The results obtained with human explants show that pravastatin specifically inhibits Rho activity in submucosal mesenchymal cells. Pravastatin also elicits ROCK inhibition, and subsequent CCN2 production in human explants and smooth muscle cells isolated from radiation enteropathy. Inhibition of type I collagen and fibronectin does occur, showing that pravastatin modulates the secretory phenotype of mesenchymal cells. Lastly, curative pravastatin administration improves radiation enteropathy in rats. This structural improvement is associated with decreased deposition of CCN2 and subsequent decreased extracellular matrix deposition.

**Conclusion:** Targeting established fibrosis with pravastatin is an efficient and safe antifibrotic strategy in radiation-induced enteropathy, and is easily transferable into the clinic.

Radiation therapy for cancer treatment faced a crucial dilemma: delivering a sufficient radiation dose for tumor control while limiting, as far as possible, normal tissue damage. Recent sophisticated irradiation modalities like three-dimensional conformal or intensity-modulated radiation therapy

increase the radiation ballistic performance in pelvic and abdominal cancer treatments. Simultaneously, the emergence of new treatment modalities based on radiochemotherapy combinations increase the risk of normal tissue toxicity (1). Thus, chronic gastrointestinal side effects (diarrhea, fecal urgency, proctitis, bleeding, fistula, etc.) still affect the daily quality of life of patients (2), and 5% to 10% develop severe intestinal toxicity characterized by a transmural fibrosis that leads to intestinal obstruction (3). This excessive accumulation of extracellular matrix (ECM) induces the loss of intestinal compliance required for aboral propulsion, and contributes to stricture formation (4). Today, surgical resection is the only effective treatment for the fibrosis, and there is a need to develop more efficient medical approaches.

Antioxidant-based antifibrotic treatments have been proposed for patients, including the combination of pentoxifylline and tocopherol (5, 6). However, the efficacy of this treatment in delayed radiation-induced intestinal toxicity is disputed (7). These inconsistent clinical reports add confusion to the old, yet unresolved controversy about the reversibility of radiation fibrosis (8). The development of high-throughput biological approaches, highlighted by the recent concept of cellular plasticity, will probably help answer this difficult question, and to identify biologically based therapeutic targets. Indeed, the study of cellular and molecular mechanisms involved in the

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**Table 1.** Animal groups and treatment schedules used

Group	Sham (15 wk)	Sham (26 wk)	19 Gy (5 wk)	19 Gy (15 wk)	19 Gy (26 wk)	19 Gy + Pravastatin (15 wk)	19 Gy + Pravastatin (26 wk)
Rats included	2	2	6	16	15	6	6
Rats analyzed	2	2	6	10	14	5	5

NOTE: Rats that died prematurely were excluded from the analysis.

persistence of human radiation enteropathy showed that severe fibrotic lesions were highly dynamic (9, 10) and associated with a high expression of the fibrogenic growth factor CCN2 in intestinal mesenchymal cells (11). In the bowel, radiation-induced fibrogenic differentiation of mesenchymal cells is characterized by cytoskeletal alterations and enhanced secretory phenotype (12, 13). In addition, an alteration of the Rho/ROCK signaling pathway in radiation enteropathy has been shown by DNA chip studies (14). Pharmacologic inhibition of this pathway using statins (Rho isoprenylation inhibition) and Y-27632 (allosteric inhibitor of ROCK) decreased CCN2 expression *in vitro*, and tended to reverse the fibrogenic differentiation of smooth muscle cells (12, 15), suggesting that inhibition of the Rho pathway and CCN2 may be a promising antifibrotic therapy.

In the present study, we postulated that pravastatin inhibited the Rho pathway and assessed this hypothesis by studying Rho and ROCK activity in human fibrotic explants. After pravastatin treatment, a specific decrease in Rho activity was detected in intestinal mesenchymal cells as well as an overall decrease in ROCK activity in protein extracts from human explants. Then, the functional consequences of Rho pathway inhibition were studied by monitoring the modulation of CCN2 expression and ECM deposition *ex vivo* and in human smooth muscle cells derived from fibrotic tissue. Finally, the antifibrotic effect of pravastatin was investigated in a rat model of radiation enteropathy and showed improvement of established radiation-induced fibrotic lesions *in vivo*. Our data show that a curative strategy using pravastatin improves radiation enteropathy through inhibition of Rho/ROCK and the subsequent decrease of CCN2 and ECM production. The data suggest that reversal of established radiation fibrosis in the gut is possible.

## Materials and Methods

**Human tissues.** Human ileum and colon tissues were collected according to the French Medical Research Council guidelines as previously described (12). Patients with prior treatment of hypercholesterolemia with statin were excluded. Ileum tissues came from two patients with small bowel occlusion subsequent to radiation enteropathy occurring at 3 and 75 months, respectively, after radiotherapy (45 Gy  $\pm$  CT, BT). Nontumoral colon tissues exhibiting radiation-induced remodeling and located within the radiation field were obtained from five patients who underwent rectal tumorectomy 6 to 8 weeks postradiotherapy (45 Gy  $\pm$  CT). Histologic assessment was carried out on tissues that were adjacent to the areas used for explant experiments. Explants (<0.5 cm<sup>2</sup>) were maintained in DMEM-Glutamax supplemented with 1% HEPES (Life Technologies) for 24 and 48 h, and were treated or not with pravastatin (Bristol-Myers Squibb) at 0.1 and 0.5 mmol/L. Samples were snap-frozen in liquid nitrogen before analysis (11).

**Human primary smooth muscle cell isolation.** Primary smooth muscle cells derived from human intestinal resection of patients with small bowel occlusion subsequent to radiation enteropathy ( $n = 3$ , radiation enteropathy-smooth muscle cells) were cultured as previously described (12) in SmGM2 medium (Clonetics) and were used at passage 4. At confluency, cells were FCS-starved for 24 h to avoid exogenous lipid contribution and incubated with 0.1, 0.5, or 1 mmol/L of pravastatin for 6 and 24 h.

**Animals and experimental procedures.** Male Wistar rats weighing 300 g at the beginning of the experimental period were obtained from the CERJ (Le Genest, France). Experiments were conducted under the French regulations for animal experimentation (Ministry of Agriculture Act no. 87-848, October 19, 1987) and received Institut de Radioprotection et de Sûreté Nucléaire ethical committee approval. Rats were anaesthetized by inhaling an air/isoflurane (TEM; Forène) mixture. A segment of the ileum was surgically exteriorized and irradiated with an X-ray machine operated at 225 kV and 17 mA with 0.5 mm copper-added filtration at a dose rate of 0.98 Gy/min. A single dose of 19 Gy was given locally on the ileum segment (6 cm), whereas the rest of the animal was shielded with a 5-mm-thick lead screen. The exteriorized segment was moistened with warm 0.9% sterile saline buffer over the course of the irradiation procedure. After irradiation, the exposed segment was returned to the abdominal cavity and the peritoneum, abdominal muscles, and skin were sutured separately.

Fifty-four animals were divided into different groups (Table 1) and treated according to the schedule (see Fig. 4A). Radiation-induced fibrotic lesions were established 5 weeks after irradiation, this time was chosen as the starting time point for pravastatin treatment and continued for 10 weeks. A clinically relevant dose of 30 mg/kg/d was used as it is known to be the standard dose for the control of hypercholesterolemia in rats (in humans, the dose required for the control of hypercholesterolemia is 40 mg/d). Rats from each experimental group were anesthetized before intestinal sampling and formol fixation.

**Quantitative histology and immunohistochemistry.** Microscopic observations were done by two independent observers. Five-micrometer alcohol, formalin, and acetic acid-fixed human specimens were stained by Masson trichrome. Optimum cutting temperature-embedded frozen explants were cut (16  $\mu$ m), postfixed in 4% paraformaldehyde, and incubated with GST-Rhotekin (0.5  $\mu$ g/ $\mu$ L) overnight at 4°C (Pierce) for *in situ* Rho activity assay. Revelation was achieved with anti-GST antibody (1:500, ab6647; AbCam) 6 h at 4°C and anti-goat Alexa 488-conjugated antibody (1:250; Molecular Probes). Fibronectin deposition was studied by immunohistochemistry with anti-fibronectin (1:400, A0245; Dako, overnight at 4°C) and anti-rabbit Alexa 546-conjugated antibody (1:250; Molecular Probes). Nuclear staining was done with To-Pro-3 iodide (1:250; Molecular Probes). Imaging was done by laser scanning confocal microscopy (Zeiss LSM510).

In rats, histologic examinations were done on three ileum segments: the irradiated segment (6 cm), and two control segments of 6 cm each, one of which was located 10 cm upstream, and one 10 cm downstream of the irradiated segment. Five-micrometer paraffin-embedded sections were stained with Masson trichrome and the collagen deposition was quantified by Image J software.<sup>7</sup> Lesions were scored dystrophic when

<sup>7</sup> ImageJ software can be downloaded from <http://rsb.info.nih.gov/ij/>.

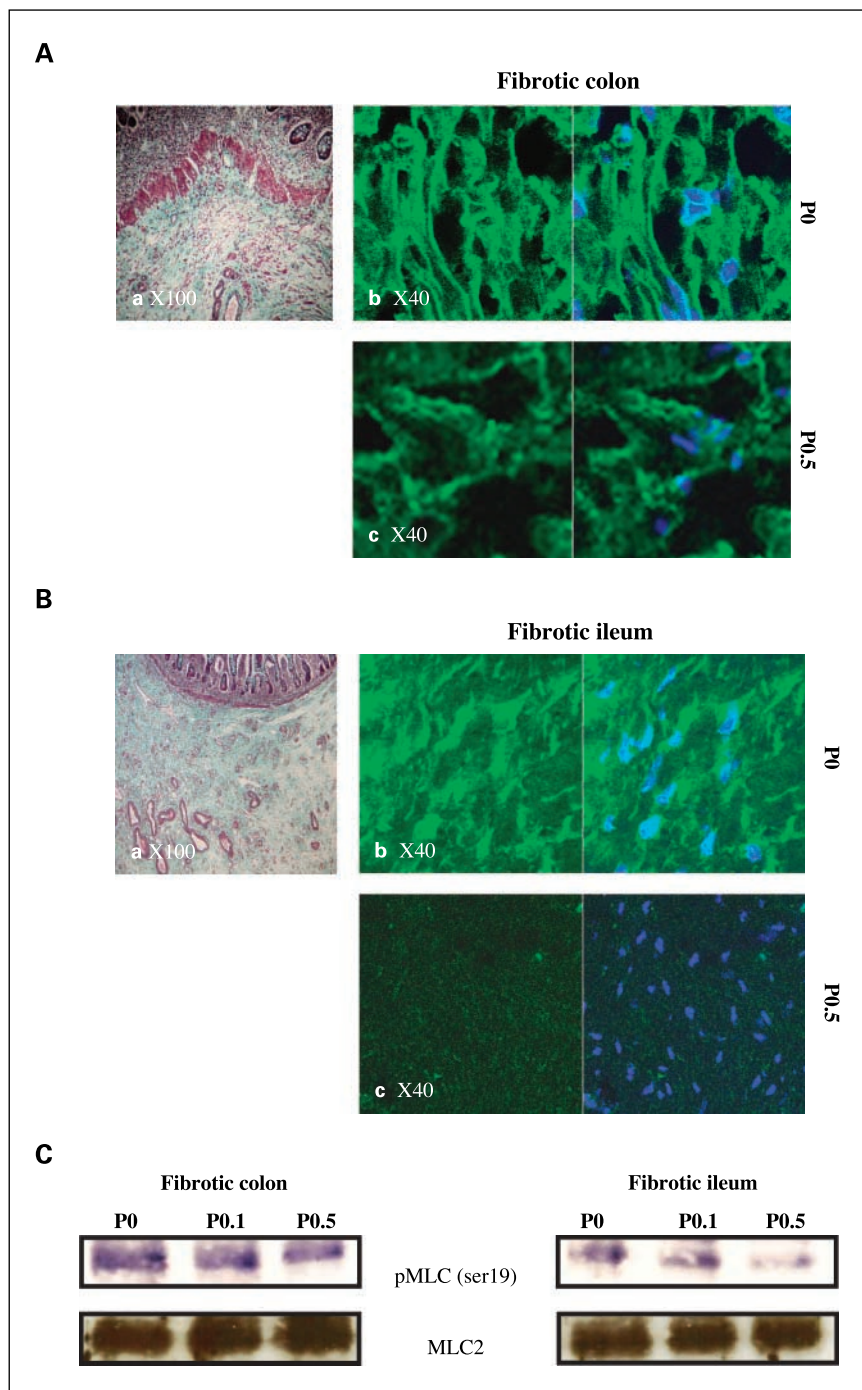
atypical villi, muscle alteration, and thickening were observed, and fibronectin when the tissue structure was replaced by dense ECM deposition. The length of dystrophy and fibronecrosis were measured using Biocom software. The CCN2 deposition was studied by immunohistochemistry as previously described (11), and quantified using Image J. The specific anti-CCN2 rabbit polyclonal antibody was provided by Dr. Cécile Martinerie (INSERM U515) and used at 1:100, overnight at 4°C.

**Real-time reverse transcription-PCR analysis.** The mRNA was isolated and analyzed as previously described (12). Primer sequences were CCN2, 5'-TCTGGGCAAACGTGTCTTC-3' (forward) and 5'-TGTGTGACGAGCC-CAAGGA-3' (reverse); collagen  $\alpha 1$ , 5'-CCTCAAGGGCTCCAACGAG-3'

(forward) and 5'-TCAATGACTGTCTTGCCCA-3' (reverse); and fibronectin, 5'-GAATATCTCGGTGCCATTTGC-3' (forward) and 5'-AGGCAT-GAAGCACTCAATTGG-3' (reverse).

**Protein isolation, immunoprecipitation, and Western blotting.** Human cells and explants were lysed in radioimmunoprecipitation assay buffer. CCN2 immunodetection by Western blot was previously described (12). Phosphorylated myosin light chain (MLC) was immunoprecipitated from 500  $\mu$ g of total explant-derived protein by rotation incubation overnight with anti-MLC2 (1  $\mu$ g, sc-15370; Santa Cruz) and protein A/G-Sepharose beads (Sigma). Beads were collected by centrifugation and washed. The immunoprecipitated phosphorylated MLC was detected with an anti-phosphorylated MLC (Ser<sup>19</sup>) antibody

**Fig. 1.** *Ex vivo* experiments were conducted with colon (A and C) and ileum (B and C). a, radiation fibrosis by Masson trichrome (green). b, intense membrane-associated Rho-GTP in submucosal fibroblasts (P0), is shut down (c) 24 h after incubation with 0.5 mmol/L of pravastatin (P0.5); nuclei (blue). C, immunoprecipitation and Western blot analysis of phosphorylated myosin light chain (pMLC) showed a decreased phosphorylated MLC level after pravastatin incubation (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5). The level of total MLC (MLC2) remained constant in colon and ileum specimens. The blots are representative of the five colon and two ileum specimens, respectively.





(1:200, CSA-410; Stressgen) by Western blotting after separation on 15% SDS-PAGE and protein transfer to nitrocellulose.

**ECM secretion analysis.** Human cells and explant supernatants were collected 24 h after pravastatin exposure, the procollagen type I (Takara, Clonetics) and fibronectin (Chemicon) secreted were measured by ELISA.

**Statistical analysis.** ELISA, quantitative reverse transcription-PCR, and *ex vivo* data were expressed as the mean  $\pm$  SEM and analyzed with Primer software using the one-way ANOVA and the Student-Newman-Keuls test. Animal experimental data were analyzed using the nonparametric Kruskal-Wallis test.

## Results

**Pravastatin decreased Rho/ROCK pathway activity in human samples.** We investigated the modulation of the Rho/ROCK pathway following pravastatin incubation of human explants. These studies were done either with intestinal ( $n = 2$ ) or colonic samples ( $n = 5$ ), as common structural and molecular characteristics have been described for colon and ileum samples with radiation-induced remodeling. Both type of samples exhibited typical lesions with characteristic transmural ECM accumulation (Fig. 1Aa and Ba). These samples were incubated *ex vivo* with 0.1 to 0.5 mmol/L of pravastatin, and modulation of Rho and ROCK activity was investigated. The results obtained here with five colon explants were similar to those obtained with two ileum explants.

To localize the cells presenting high Rho activation, an *in situ* Rho activity assay was done in thick cryosections as previously described (16). Activated Rho proteins were detected at the cell membrane of epithelial cells, endothelial cells of the submucosal vessels (data not shown), and submucosal mesenchymal cells (Fig. 1Ab and Bb). Rho activation was difficult to monitor properly in the muscular layers due to the high density of fibers. Modulation of Rho activity was investigated after treatment with 0.5 mmol/L of pravastatin, and showed a specific decreased staining of membrane-associated Rho-GTP in submucosal fibroblasts (Fig. 1Ac and Bc), whereas no variation was detected in epithelial and endothelial cells. ROCK activity was indirectly assessed in explants' crude protein extracts by examining MLC phosphorylation, a well-known target of ROCK. Pravastatin treatment decreased MLC phosphorylation in both colon and ileum explants, whereas total MLC content remained stable (Fig. 1C). These results show that pravastatin decreased Rho and ROCK activity *ex vivo*.

**Pravastatin induces CCN2 inhibition *ex vivo* and *in vitro*.** We hypothesized that Rho/ROCK inhibition would subsequently inhibit CCN2 expression. When human colon and ileum explants were incubated *ex vivo* with pravastatin, a significant decrease in CCN2 mRNA levels was observed after 24 h of exposure to 0.1 to 0.5 mmol/L of pravastatin ( $P < 0.05$ ; Fig. 2A), whereas 48 h exposure to 0.5 mmol/L of pravastatin was required to inhibit CCN2 protein expression (Fig. 2B). We confirmed this pravastatin-induced CCN2 inhibition *in vitro* in primary human smooth muscle cells isolated from human radiation enteropathy. Interestingly, 6 h incubation with 0.1 to 1 mmol/L of pravastatin was sufficient to significantly reduce CCN2 mRNA and protein levels ( $P < 0.05$ ; Fig. 2C and D).

**Pravastatin induces inhibition of collagen and fibronectin deposition *ex vivo* and *in vitro*.** Next, we investigated the downstream effect of pravastatin inhibition on the Rho/ROCK/CCN2 cascade by studying two important components of the

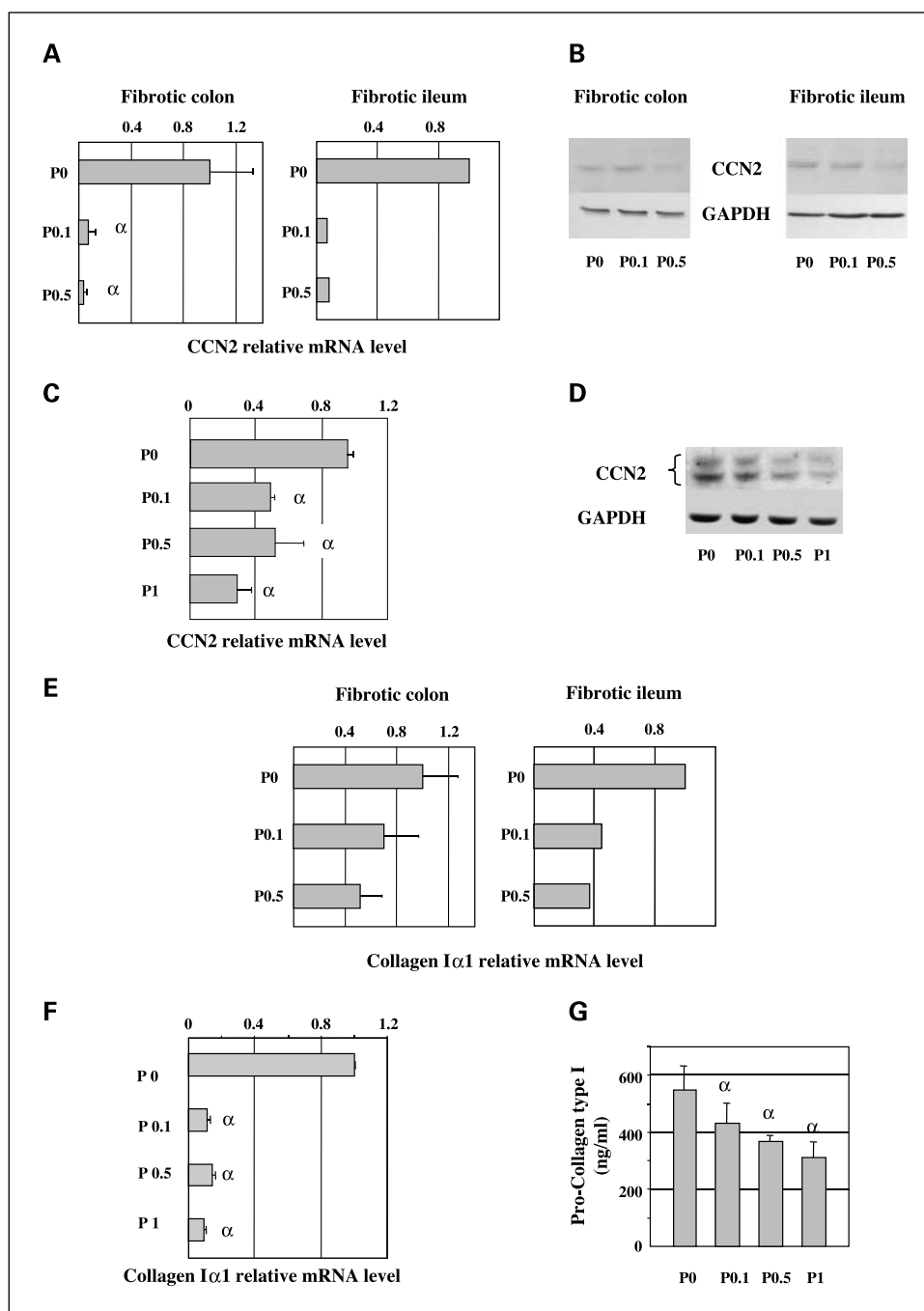
fibrotic ECM in the intestine, i.e., type I collagen and fibronectin. Exposure to increasing doses of pravastatin decreased type I collagen and fibronectin mRNA levels *ex vivo* and *in vitro* ( $P < 0.05$ ; Figs. 2E and 3A and Figs. 2F and 3D, respectively). The decrease in collagen content was confirmed in cell supernatants (Fig. 2G) and the decrease in fibronectin content was specifically confirmed both *in situ* (Fig. 3B-C) and *in vitro* in cell supernatants (Fig. 3E).

**Pravastatin improves delayed radiation enteropathy in rats.** In order to investigate the efficacy of pravastatin for the treatment of radiation-induced intestinal fibrosis, radiation enteropathy was modeled in rats. Five weeks after irradiation, histologic examinations showed typical radiation-induced fibrotic lesions with dystrophic and fibronectrotic zones in the irradiated group. In dystrophic areas, lesions consisted of atypical villi, muscle alteration, and thickening (Fig. 4C). In fibronectrotic areas, the tissue structure was replaced by dense ECM deposits (Fig. 4C). Fifteen and 26 weeks after irradiation, epithelial border recovery occurred in dystrophic areas, but muscular lesions worsened with thick and edematous muscular layers (Fig. 5ae). Fibronectrotic areas remained with dense ECM deposition and intense inflammatory cell infiltration (Fig. 5cg). No fibrotic remodeling was observed in the out-field ileum segments.

In the irradiated group treated with pravastatin, the severity of muscular alterations decreased 15 weeks after irradiation. The length of the dystrophic lesions decreased by 34% ( $P = 0.09$ ) and muscular structure recovery was observed (Fig. 5b). In fibronectrotic areas, no significant structural improvement was observed in the pravastatin versus control group (Fig. 5d). One group of animals was kept until week 26, 11 weeks after pravastatin treatment completion, to investigate the possible occurrence of a recall effect. Interestingly, both dystrophic and fibronectrotic lesions were recovered in this group (Fig. 5fh). Indeed, the muscularis propria seemed to be nearly normal, whereas submucosal vessel hyalinization was the only obvious sign of fibrotic remodeling in the remaining dystrophic zones (Fig. 5f). In addition, partial re-epithelialization and decreased thickening of the intestinal wall occurred in fibronectrotic areas. However, necrotic tissue still replaced the muscular layers (Fig. 5h).

**Pravastatin treatment controls the expression of CCN2 and ECM in rats.** Our previous experiments suggest that pravastatin treatment would inhibit the expression of the fibrogenic growth factor CCN2, but this had not been shown *in vivo*. Thus, CCN2 deposition was monitored by densitometric analysis of the CCN2 staining and showed a significant decrease in the pravastatin groups treated for 15 weeks in both the dystrophic ( $P = 0.003$ ) and fibronectrotic ( $P = 0.0001$ ) areas, and even more importantly, 26 weeks after irradiation ( $P = 0.0001$ ; Fig. 6A and B). CCN2 immunohistochemistry showed strong CCN2 staining in submucosal mesenchymal cells, vessels, and muscular layers (Fig. 6C), which decreased in the pravastatin-treated group. This CCN2-decreased deposition was associated with improvement of the intestinal structure and supports the idea that pravastatin inhibits the Rho/ROCK pathway, and subsequently, CCN2 expression in our rat model. Collagen deposition was quantified and no significant modulation was observed at 15 weeks (Fig. 6D). But 26 weeks after radiation, a 2.7-fold decrease in collagen deposition was measured in the pravastatin-treated group ( $P = 0.0001$ ; Fig. 6D), showing that pravastatin inhibits ECM deposition.

**Fig. 2.** *A*, real-time reverse transcription-PCR analysis of CCN2 mRNA level in human explants after incubation with pravastatin for 24 h (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5). 18S RNA was used for normalization;  $\alpha$ ,  $P < 0.05$ . Statistical analysis was not done on ileum samples that were collected from two patients. *B*, Western blot analysis of CCN2 in human explants after incubation with pravastatin for 48 h (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The blots are representative of the five colon and two ileum specimens, respectively. Real-time reverse transcription-PCR (*C*) and Western blot analysis (*D*) of CCN2 expression in human smooth muscle cells derived from radiation enteropathy after incubation with pravastatin for 6 h (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5; 1 mmol/L, P1;  $\alpha$ ,  $P < 0.05$ ). The blots are representative of three independent experiments, respectively. *E* and *F*, real-time reverse transcription-PCR analysis of COL1A1 mRNA level after incubation with increasing doses of pravastatin for 24 h (*E*) in human explants and for 6 h (*F*) in smooth muscle cells (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5; 1 mmol/L, P1;  $\alpha$ ,  $P < 0.05$ ). *G*, the secretion of type I procollagen was monitored by ELISA in smooth muscle cell supernatant after incubation with pravastatin for 24 h (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5; 1 mmol/L, P1;  $\alpha$ ,  $P < 0.05$ ).



## Discussion

The present preclinical studies propose a cellular and molecular rationale for using pravastatin as antifibrotic therapy in irradiated gut. They further propose a novel and clinically relevant function for pravastatin as a modulator of the fibrogenic secretory phenotype of the mesenchymal compartment in fibrotic tissue. Thus, besides its beneficial action on immune and vascular function, curative pravastatin administration inhibits the chronically activated Rho/ROCK/CCN2/ECM cascade *ex vivo* in human samples. In addition, the curative administration of pravastatin improves established radiation enteropathy lesions in rats. Our data suggest that the

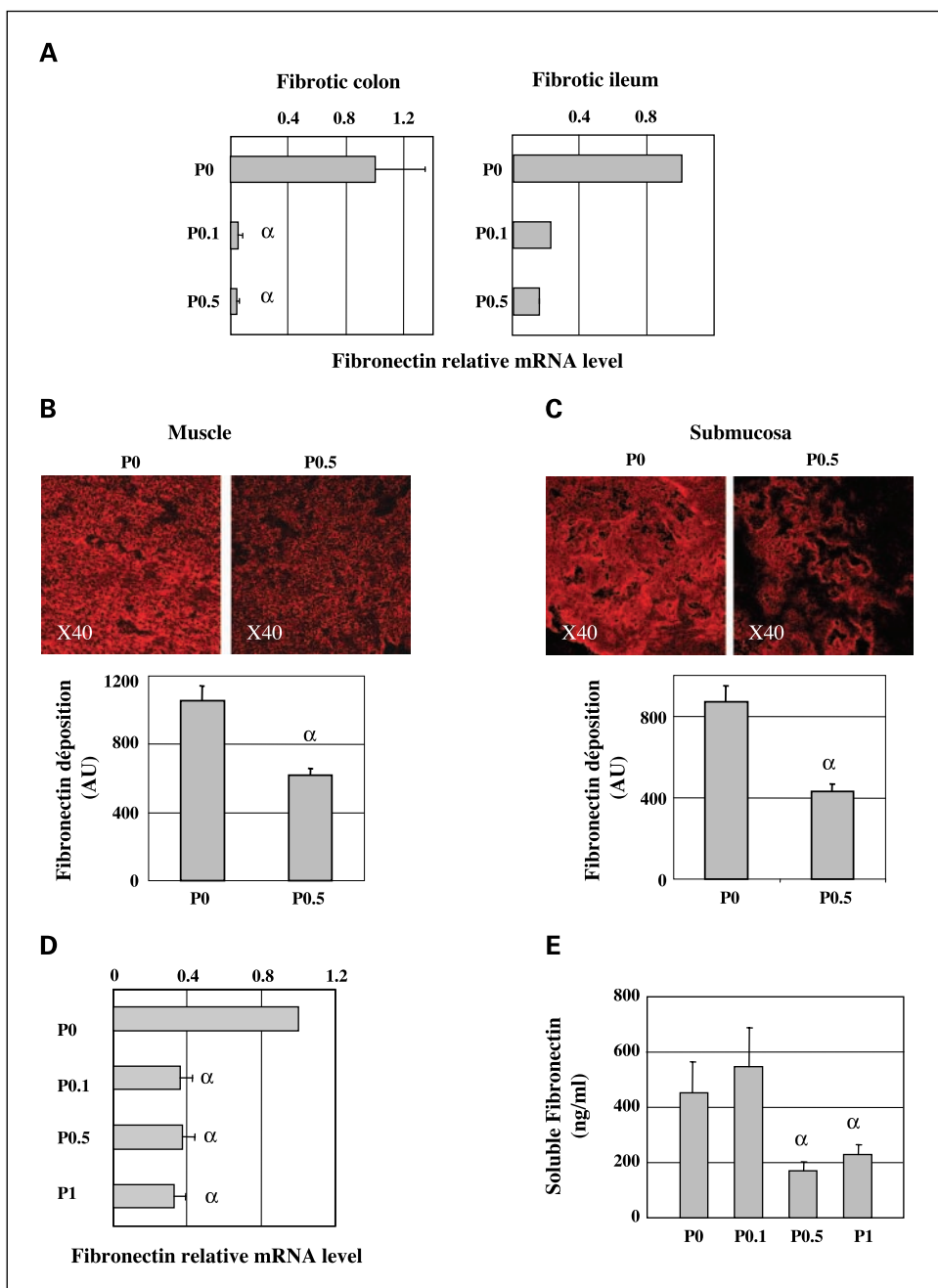
pravastatin-based strategy is an efficient and safe antifibrotic therapy, easily transferable into the clinic to improve the quality of life of long-term cancer survivors without interfering with prior anticancer treatment. Furthermore, this curative strategy is applicable to treat delayed radiation injury in case of radiation accidents or acts of terrorism (17).

The biochemical maintenance of radiation fibrosis is a complex process that depends on continuous and integrated activation loops that involve cell differentiation and cross-talk between the various cellular components of the tissue within the matrix (18). In this context, targeting one central pathway involved in vascular, immune, and stromal pathogenic response would provide an efficient antifibrotic strategy, and

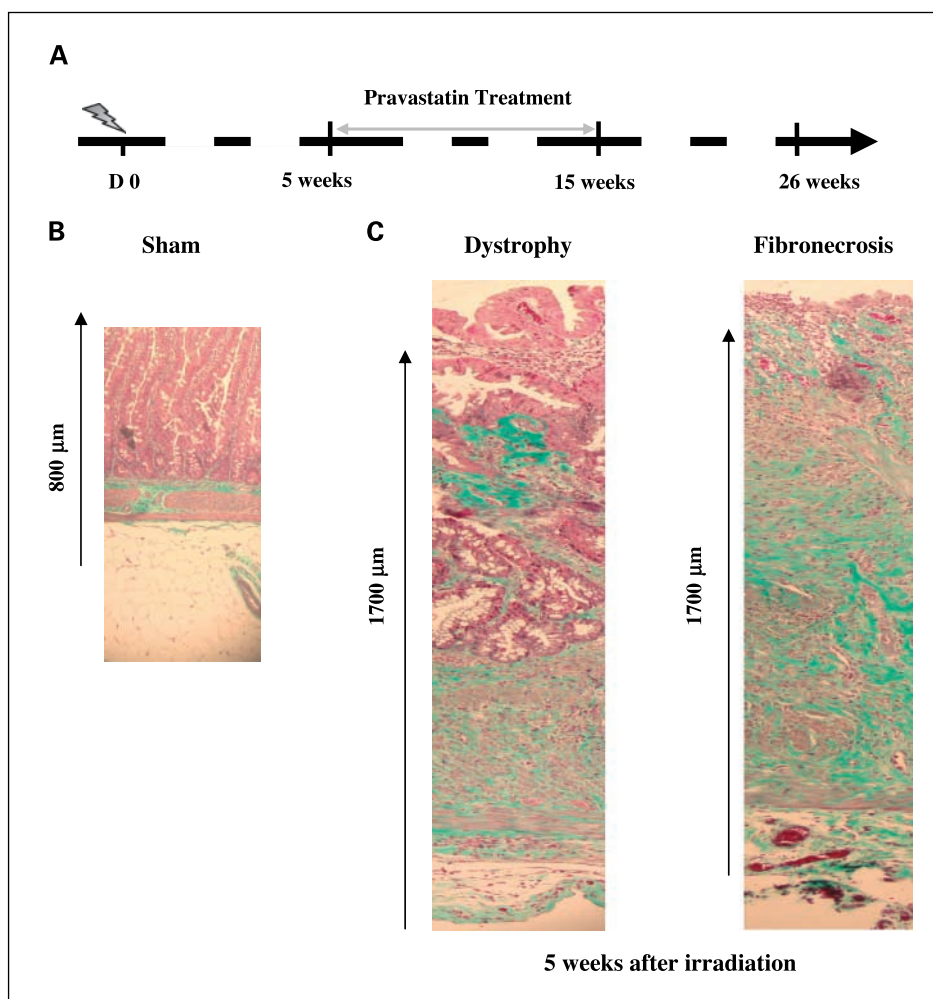
this was the ultimate purpose of the present study targeting Rho activation with pravastatin. Pravastatin is a natural hydrophilic statin that was chosen among the various statins available for its ileal specificity. Indeed, pravastatin uptake is thought to occur specifically in the ileum via bile acids (19, 20). Statins have been extensively prescribed for their cholesterol-lowering properties over the past 10 years; however, it has become increasingly evident that they have beneficial effects beyond their lipid-lowering action. Greenwood et al. compiled evidence that the pleiotropic action of statins were mediated by inhibition of the production of isoprenoid residues and subsequent modulation of posttranslational protein prenylation, including that of Rho (21). Rho proteins are small GTPases acting as molecular switches to control a wide range of

cellular functions like cell adhesion, formation of stress fibers, and cellular contractility through the reorganization of actin-based cytoskeletal structures. These functions are accomplished specifically via their effectors, the ROCKs (22).

The Rho pathway is known to control both vascular (23) and immune functions (21), and these functions have been elucidated using a range of pharmacologic inhibitors. The most prominent of these inhibitors are the statins. Indeed, statins modulate the TH1/TH2 balance via Rho inhibition, thus interfering with chronic inflammation (21, 24). They also regulate the endothelial barrier functions, inflammation and transendothelial leukocyte migration, platelet activation, thrombosis, and oxidative stress, as well as the homeostasis of vascular smooth muscle cells (25–28). The contribution of



**Fig. 3.** A and D, real-time reverse transcription-PCR analysis of fibronectin mRNA level after incubation with increasing doses of pravastatin for 24 h (A) in human explants and for 6 h (D) in smooth muscle cells (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5;  $\alpha$ ,  $P < 0.05$ ). B and C, *in situ* fibronectin deposition was quantified in colonic muscularis propria (B) and submucosa (C) after incubation with increasing doses of pravastatin for 24 h (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5;  $\alpha$ ,  $P < 0.05$ ). E, the secretion of fibronectin was monitored by ELISA in smooth muscle cells supernatant after incubation with pravastatin for 24 h (0 mmol/L, P0; 0.1 mmol/L, P0.1; 0.5 mmol/L, P0.5; 1 mmol/L, P1;  $\alpha$ ,  $P < 0.05$ ).



**Fig. 4.** A, pravastatin curative treatment schedule in experimental radiation enteropathy. Masson trichrome staining of unirradiated (B, sham) and irradiated (C) ileum of rats 5 wk after irradiation (dystrophy, fibronecrosis), before pravastatin treatment (magnification,  $\times 100$ ).

the vascular compartment to radiation enteropathy is undisputable (29, 30); thus, statin efficacy in radiation enteropathy was expected but not investigated *in vivo* until now. The present study is the first to report a significant improvement of the intestinal structure after pravastatin administration in a rat model of radiation enteropathy, and confirms the relevance of these findings for human pathology using human samples. These experiments in the gut confirm the results previously obtained by Williams et al. In William's study, conducted on mice, the beneficial action of lovastatin on radiation-induced pulmonary fibrosis was reported to be associated with decreased collagen deposition attributed to the classic anti-inflammatory action of lovastatin such as decreased macrophage and lymphocyte recruitment (31). In the present study, another explanation for decreased collagen deposition is proposed based on the direct ability of pravastatin to decrease ECM and CCN2 expression secreted by the mesenchymal compartment through alteration of the Rho pathway.

For a long time, the role of the mesenchymal compartment was thought to be secondary. However, in the intestine, the obstruction is caused by the pathologic accumulation of ECM. This abnormal collagen accumulation is achieved by specialized smooth muscle cells (32) that exhibit chronic fibrogenic differentiation. In radiation enteropathy, the fibrogenic differentiation is characterized by cytoskeletal alterations and

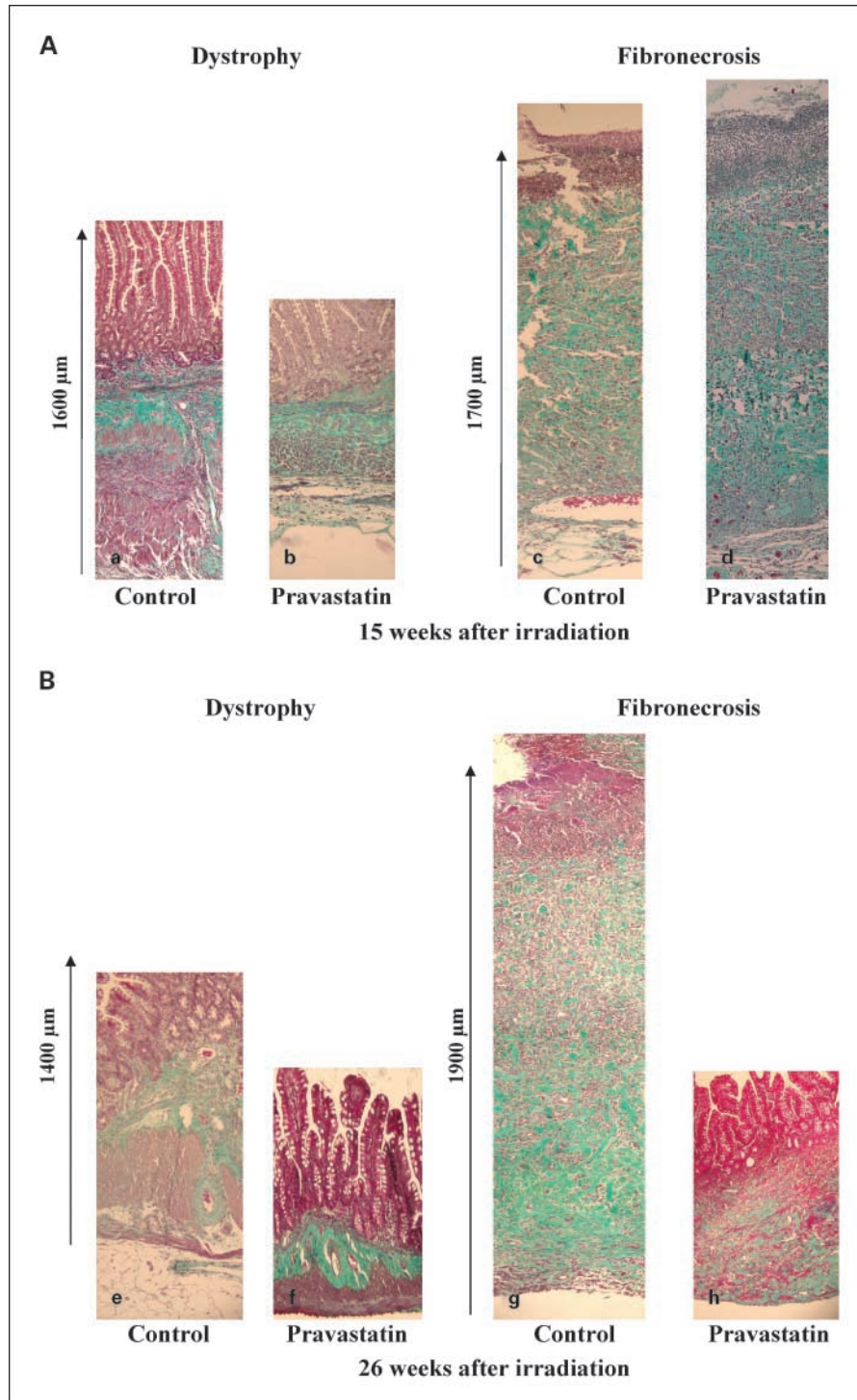
enhanced secretory phenotype (i.e., ECM components and profibrotic growth factors production; refs. 11, 12, 15). The maintenance of this pathologic differentiation was thought to be controlled by transforming growth factor- $\beta 1$  (33). Yet, in human delayed radiation enteropathy, transforming growth factor- $\beta 1$  expression is low, suggesting that activation of an alternative cascade during the chronic phase of the pathology occurs. Indeed, elevated CCN2 levels have been found (11), suggesting that during the chronic phase of radiation enteropathy, CCN2 triggers pathologic ECM production and might constitute a specific and efficient antifibrotic target (34). In addition, we recently showed that the Rho pathway controls the chronic production of the fibrogenic growth factor CCN2 and abnormal accumulation of the ECM in intestinal smooth muscle cells derived from radiation enteropathy (12). The fact that statins are potent pharmacologic inhibitors of the Rho pathways provides an opportunity for therapeutic intervention targeting the Rho/CCN2 cascade. Until now, modulating the activation of the Rho pathway and subsequent CCN2 production using statins was reported *in vitro* (15, 35), but in this study, this strategy was validated *ex vivo* in human samples. Direct evaluation of Rho and ROCK activity was done in human samples. In the rat model, we monitored the modulation of CCN2, a direct target of the Rho/ROCK pathway (12, 36) and found that CCN2 decreased in pravastatin-treated



animals. In addition, the improvement of the intestinal structure observed on week 26, i.e., 11 weeks after pravastatin treatment completion, suggests that a chronic activation loop responsible for the maintenance of fibrosis was disrupted by the treatment. Our data suggests that this fibrogenic activation loop is at least partly controlled by the Rho pathway, the subsequent activation of CCN2 and stimulation of collagen and fibronectin deposition.

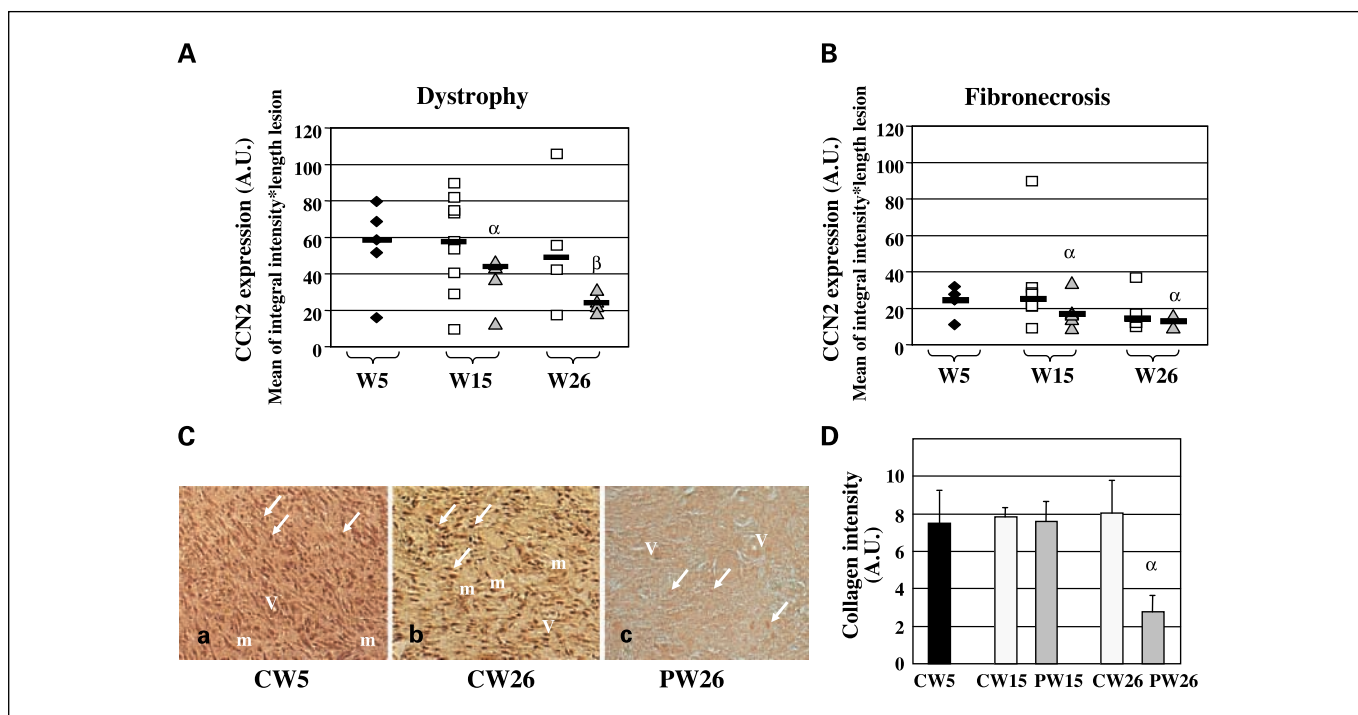
Classically, improvement of fibrosis is associated with the elimination of fibrosis-activated mesenchymal cells by apopto-

sis or necrosis (37). The occurrence of apoptosis/necrosis in our model remains to be investigated, but we propose that tissue restoration might involve phenotypic reversion. Indeed, the inhibition of CCN2, collagen, and fibronectin expression in primary smooth muscle cells derived from radiation enteropathy by pravastatin suggests a partial reversion of the fibrogenic phenotype. Such reversal has already been reported by our group in various cellular models derived from the intestine using a ROCK inhibitor (12, 13), and in skin myofibroblasts using antioxidant therapy (38). These results challenge the



**Fig. 5.** Masson trichrome staining in irradiated groups (*a* and *e*, dystrophy; *c* and *g*, fibronecrosis) and pravastatin-treated groups (*b* and *f*, dystrophy; *d* and *h*, fibronecrosis) 15 (*A*) and 26 (*B*) weeks after irradiation (magnification,  $\times 100$ ).





**Fig. 6.** Quantification of CCN2 deposition in dystrophic (A) and fibronectic (B) areas in pravastatin-treated ( $\square$ ) versus untreated ( $\triangle$ ) rats 5 wk (W5), 15 wk (W15) and 26 wk (W26) after irradiation. Points, means of CCN2 deposition measured for each animal ( $n = 6$  slides/animal), values are normalized to lesion length; bars, median ( $\alpha$ ,  $P = 0.003$ ;  $\beta$ ,  $P < 0.0001$ ). C, CCN2 immunolocalization (brown) in untreated rats 5 wk (CW5) and 26 wk (CW26) after irradiation and pravastatin-treated rats 26 wk (PW26) after irradiation, bright-field photomicrograph ( $\times 100$ ). The brown dots are CCN2-positive mesenchymal cells (arrows) and vessels (V) in fibronectic zones and showed matrix-associated CCN2 (m). D, quantification of collagen deposition in pravastatin-treated versus untreated rats 5 wk (W5), 15 wk (W15) and 26 wk (W26) after irradiation ( $\alpha$ ,  $P < 0.0001$ ).

conventional wisdom of a radiation-induced terminal differentiation of mesenchymal cells (39, 40). In addition, this important finding is not restricted to radiation-induced fibrosis, but was also observed in scleroderma (41), and in hepatic (37) and kidney fibrosis (42). The plasticity of mesenchymal cells seems to be more important than previously thought, thereby suggesting options to treat fibrotic pathologies in general.

The development of curative antifibrotic strategy is nowadays highly expected by both patients and physicians (8). Indeed, the high efficacy of current anticancer treatments increase the patients' overall survival, but also increase late complications, especially in the gut (2). Yet, the possibility of radiation fibrosis reversion was still doubted for clinical methodologic reasons and lack of biological evidence (8). Here, we propose a molecular rationale for the use of pravastatin as an antifibrotic therapy in the gut. Pravastatin inhibits the Rho/ROCK/CCN2/ECM cascade in human samples *ex vivo* and improves intestinal radiation-induced fibrosis *in vivo*. Although the additional action of pravastatin on vascular and immune function is not

excluded, the present study showed that targeting fibrogenic differentiation is an efficient antifibrotic strategy in the intestine. Pravastatin is a particularly attractive therapeutic agent because it is administered orally, its safety profile is good (only few rhabdomyolysis cases reported), and its uptake is high in the ileum. In addition, we recently showed in mitigation experiments that pravastatin protects normal intestine from radiation damage without interfering with the anticancer action of irradiation in experimental models *in vitro* and *in vivo* (43). Thus, the antifibrotic efficacy of pravastatin will soon be studied in a phase II randomized clinical trial at our institution.

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