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Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis

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Serum paraoxonase (PON1) is an esterase that is associated with high-density lipoproteins (HDLs) in the plasma; it is involved in the detoxification of organophosphate insecticides such as parathion and chlorpyrifos[1–3]. PON1 may also confer protection against coronary artery disease by destroying pro-inflammatory oxidized lipids present in oxidized low-density lipoproteins (LDLs)[4–8]. To study the role of PON1 *in vivo*, we created *PON1*-knockout mice by gene targeting. Compared with their wild-type littermates, PON1-deficient mice were extremely sensitive to the toxic effects of chlorpyrifos oxon, the activated form of chlorpyrifos, and were more sensitive to chlorpyrifos itself. HDLs isolated from PON1-deficient mice were unable to prevent LDL oxidation in a co-cultured cell model of the artery wall, and both HDLs and LDLs isolated from *PON1*-knockout mice were more susceptible to oxidation by co-cultured cells than the lipoproteins from wild-type littermates. When fed on a high-fat, high-cholesterol diet, *PON1*-null mice were more susceptible to atherosclerosis than their wild-type littermates.

PON1-knockout mice were produced by targeted disruption of exon 1 of the *PON1* gene (Fig. 1a). Mice homozygous for the mutation were identified by Southern blot analysis (Fig. 1b). The *PON1* mutant allele was transmitted in a mendelian fashion, and *PON1* homozygous mutant mice were normal in their appearance and body weight. We detected no PON1 protein in plasma samples from homozygous mutant mice by immunoblotting using a polyclonal antiserum prepared against murine PON1 (Fig. 1c). Wild-type ($PON1^{+/+}$) mice on average had 367 units l^{-1} of paraoxonase activity in plasma, whereas *PON1* homozygous mutant ($PON1^{-/-}$) mice had no detectable paraoxonase activity in their plasma (Fig. 2a). Heterozygous ($PON1^{+/-}$) mice had ~50% of the paraoxonase activity of their wild-type littermates (data not shown). *PON1*-knockout mice also showed an ~85% decrease in plasma arylesterase activity using phenylacetate as substrate, indicating that most, but not all, of this activity is due to PON1 (data not shown).

PON1 is important in the detoxification of organophosphate insecticides. Injection of purified PON1 protects against organophosphate toxicity in rodents[9–11], and common polymorphisms in humans of the *PON1* gene dramatically influence the activity of the enzyme towards various organophosphate substrates[12,13]. To study the role *in vivo* of PON1 in detoxifying these compounds, we examined the sensitivity of $PON1^{-/-}$ mice towards chlorpyrifos (CPS) and its oxon (CPO). CPO is a potent cholinesterase inhibitor and is the activated metabolite of CPS; it is a substrate for PON1 hydrolysis. Our $PON1^{-/-}$ mice had only 2% of the plasma chlorpyrifos-oxonase activity of $PON1^{+/+}$ mice (Fig. 2b), suggesting that PON1 is the predominant enzyme that hydrolyses CPO in the circulation. To investigate its toxicity, three dosages of CPO (1.5, 3, and 6 mg kg^{-1} body weight) were applied to *PON1* wild-type and null mice. Four hours after treatment, the activity of acetylcholinesterase (AChE) in brain and diaphragm were determined as indicators of toxicity. At the lowest dose (1.5 mg kg^{-1}), CPO did not affect AChE activity in wild-type mice but reduced it by 80 and 74%

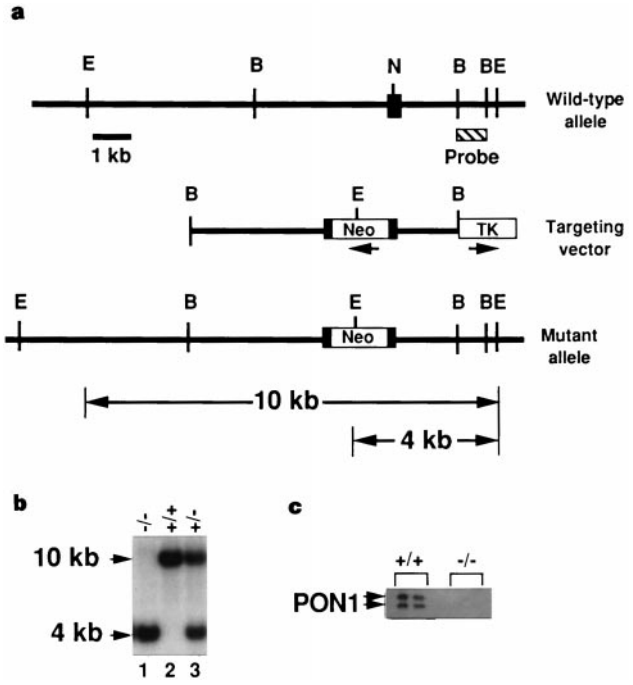

Figure 1 Targeted disruption of the *PON1* gene. **a**, Partial restriction maps of the wild-type *PON1* locus, targeting vector and mutant allele. For gene targeting of the *PON1* locus, exon 1 (filled box) is disrupted by insertion of a positive-selection marker (MC1 Neo). The MC1-TK (thymidine kinase) gene was used for negative selection. The external probe used for Southern blot analysis is shown in the hatched box. B, *Bgl*II; E, *Eco*RI, N, *Nhe*I. **b**, Southern blot analysis of DNA isolated from: lane 1, a mouse homozygous for the targeted allele; lane 2, a mouse homozygous for the wild-type allele; and lane 3, a heterozygous mouse. The wild-type and mutated genes gave 10.0-kb and 4.0-kb fragments, respectively, after *Eco*RI digestion and hybridization with the external probe. **c**, Targeted mice are deficient in PON1 protein. Immunoblotting of PON1 showed the lack of immunoreactive PON1 in the plasma of $PON1^{-/-}$ mice.

Table 1 Plasma lipoprotein, lipid and PON1 levels in wild-type, PON1 heterozygous and *PON1* null mice

	Total cholesterol	VLDL/LDL cholesterol	HDL cholesterol	Unesterified cholesterol	Triglycerides	Free fatty acids	Relative PON1 activity*
Female, on chow diet							
PON1$^{+/+}$ ($n = 11$)	110 ± 7	13 ± 3†	98 ± 5	17 ± 2	40 ± 7	75 ± 4	100
PON1$^{+/-}$ ($n = 8$)	101 ± 11	11 ± 3‡	90 ± 8	16 ± 3	37 ± 8	77 ± 6	50 ± 4
PON1$^{-/-}$ ($n = 16$)	122 ± 8	19 ± 2†‡	103 ± 6	22 ± 2	66 ± 11	81 ± 6	0 ± 1
Male, on chow diet							
PON1$^{+/+}$ ($n = 10$)	126 ± 8	19 ± 3	107 ± 7	27 ± 3	96 ± 24	79 ± 5	70 ± 9
PON1$^{+/-}$ ($n = 9$)	147 ± 10	21 ± 3	125 ± 9	28 ± 3	93 ± 15	89 ± 5	40 ± 5
PON1$^{-/-}$ ($n = 16$)	136 ± 6	16 ± 2	119 ± 5	27 ± 2	97 ± 14	82 ± 3	0 ± 1
Female, on high-fat diet							
PON1$^{+/+}$ ($n = 6$)	256 ± 22	198 ± 23	58 ± 8	51 ± 4	4 ± 1	36 ± 3	38 ± 4
PON1$^{-/-}$ ($n = 6$)	261 ± 22	197 ± 21	64 ± 10	50 ± 6	3 ± 1	33 ± 1	0 ± 2

Lipid levels are given in mg dl^{-1} ± s.e. Values for lipid levels on chow diets were from mice of 50% C57BL/6 and 50% 129/SvJ genetic background; lipid levels of mice on an atherogenic, high-fat diet were from animals of 87.5% C57BL/6 and 12.5% 129/SvJ genetic background.
* PON1 activities were determined using arylesterase as substrate and are given as a percentage ± s.e. compared to female wild-type mice fed a chow diet, after subtracting the arylesterase activity in *PON1*$^{-/-}$ mice. Values are means ± s.e. from sample sizes of between 4 to 8 mice per group. All data were from mice of 87.5% C57BL/6 and 12.5% 129/SvJ genetic background.
† Student's *t*-test: $P \leq 0.05$.
‡ Student's *t*-test: $P < 0.05$.

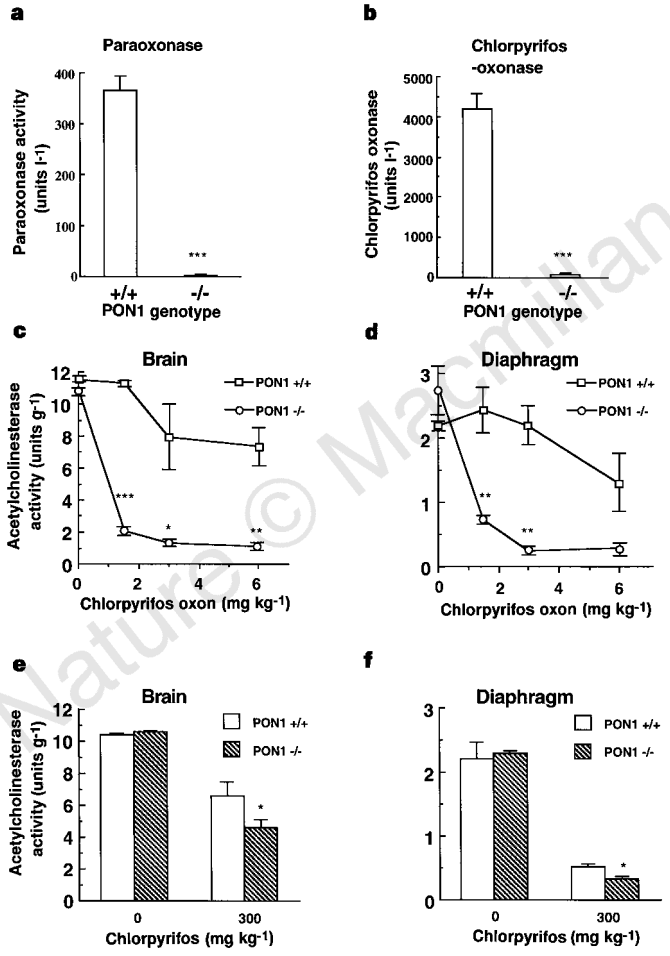

Figure 2 *PON1* null mice are more sensitive to the toxic effects of chlorpyrifos oxon and chlorpyrifos. **a**, Plasma paraoxonase, and **b**, chlorpyrifos oxonase activities of wild-type and *PON1* null mice. There were 10 and 9 animals in the *PON1*$^{+/+}$ and *PON1*$^{-/-}$ groups, respectively. **c**, **d**, Acetylcholinesterase activity in brain (**c**) and diaphragm (**d**) samples from *PON1*$^{+/+}$ and *PON1*$^{-/-}$ mice treated with chlorpyrifos oxon. There were 3 animals in each treatment group. **e**, **f**, Acetylcholinesterase activity in brain (**e**) and diaphragm (**f**) samples from *PON1*$^{+/+}$ and *PON1*$^{-/-}$ mice treated with chlorpyrifos. There were 4–6 mice in each group. For **a**–**f**, data are the means obtained from several animals. Error bars indicate standard errors; Student's *t*-test was used for statistical analysis. Asterisks indicate significant differences between *PON1*$^{-/-}$ and *PON1*$^{+/+}$ groups at the significance level of $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.0001$, respectively.

in brain and diaphragm, respectively, in *PON1*-null mice (Fig. 2c, d). At a dose of 3 mg kg^{-1}, CPO inhibited brain AChE activity in *PON1*$^{+/+}$ animals by 31%, whereas it killed *PON1*$^{-/-}$ mice within 4 h of exposure. AChE activity in both the brains and diaphragms of *PON1*$^{-/-}$ mice was significantly lower than that in *PON1*$^{+/+}$ mice. All of the *PON1*$^{-/-}$ mice that received 6 mg kg^{-1} of CPO died within 2 hours of exposure, whereas the *PON1*$^{+/+}$ mice that received the same dose of CPO survived and their AChE activity was mildly inhibited. *PON1*$^{+/+}$ mice that received the 6 mg kg^{-1} or lower doses of CPO showed no symptoms of CPO poisoning. Acute symptoms, including weakness, lacrimation, convulsions and difficulty in breathing, developed in *PON1* null mice 1–2 h after CPO exposure.

CPS is not itself a substrate for hydrolysis by PON1 and must first be converted to CPO by cytochrome P450 enzyme systems. We therefore did a toxicology test on *PON1* null mice for increased sensitivity to CPS. A high dose of CPS (300 mg kg^{-1}) reduced the AChE activity (in brain and diaphragm tissue) of *PON1*$^{-/-}$ mice significantly more than in *PON1*$^{+/+}$ mice (Fig. 2e, f), although the difference in CPS toxicity between wild-type and *PON1* null mice was less than the difference in CPO toxicity. We conclude that *PON1* null mice are sensitive to the toxic effects of CPO as well as of CPS and that PON1 is responsible for detoxification of organophosphorus pesticides. Therefore, genetic variations in PON1 activity are likely to affect the response of humans to accidental exposure.

As human population studies have revealed an association between PON1 expression and lipoprotein levels[7], we studied the effects of PON1 deficiency on plasma lipoproteins. We found no significant difference in the amount of lipoproteins in male mice of different *PON1* genotypes but, compared with female *PON1*$^{+/+}$ or *PON1*$^{+/-}$ mice, female *PON1*$^{-/-}$ mice had a moderate increase in plasma cholesterol and triglyceride associated with very-low-density lipoproteins (VLDLs) and LDLs (Table 1).

HDLs protect LDLs against oxidation in a co-culture model of the artery wall consisting of a monolayer of aortic endothelial cells overlying the extracellular matrix produced by a layer of smooth muscle cells underneath[14,15]. In this microenvironment, exogenously added LDLs become modified by oxidation in the subendothelial matrix; the oxidized LDL then induces the synthesis and secretion of monocyte chemotactic protein-1 (MCP-1) which can be recovered and assayed by its ability to induce monocyte adhesion and transmigration[14]. Co-incubation of human LDLs with *PON1*$^{+/+}$ HDLs decreased monocyte transmigration by 57% compared with incubation with LDL alone ($P < 0.05$) (Fig. 3a). Co-incubation of LDLs with *PON1*$^{+/-}$ HDLs reduced monocyte transmigration by 38% (data not shown) compared with LDL alone, whereas incubation of LDLs with *PON1*$^{-/-}$ HDLs did not affect monocyte transmigration, indicating that there is a dose-dependent effect of *PON1* expression on the ability of HDL to prevent LDL oxidation. When

PON1$^{-/-}$ HDLs were incubated with co-culture cells without LDL or oxidized phospholipids, they also induced monocyte chemotactic activity, whereas *PON1*$^{+/+}$ HDLs did not (Fig. 3a). However, the activity induced by *PON1*$^{-/-}$ HDL alone was significantly lower than in the human LDL plus *PON1*$^{-/-}$ HDL group ($P < 0.0001$) (Fig. 3a), indicating that HDLs from *PON1* null mice cannot prevent LDL oxidation and are also pro-inflammatory.

To investigate lipoprotein oxidation and the inflammatory response this induces, we measured the increase in lipid hydroperoxides (LOOH) and in MCP-1 in the medium after treatment of co-cultured cells with HDLs isolated from wild-type and *PON1*-knockout mice (Fig. 3b–d). We found that pretreatment overnight of co-cultured cells with *PON1*$^{+/+}$ HDLs blocked LOOH accumulation in human LDLs (Fig. 3b) and greatly reduced the amount and chemotactic activity of MCP-1 (Fig. 3c, d) in conditioned medium compared with the human LDL-alone group, whereas pretreatment with *PON1*$^{-/-}$ HDLs did not (Fig. 3b–d).

When *PON1*$^{-/-}$ HDLs were supplemented with 4 μg ml^{-1} purified human PON1 protein, paraoxonase activity on these HDLs increased from zero in sham-treated *PON1*$^{-/-}$ HDL to 9.8 units ml^{-1}. Pretreatment of co-cultured cells with these supplemented *PON1*$^{-/-}$ HDLs restored the ability of the HDLs to block LOOH accumulation in human LDLs (Fig. 3b) and also decreased the amount of MCP-1 (Fig. 3c) and its chemotactic activity (Fig. 3d) in conditioned medium. Co-cultured cells pretreated with human PON1 also prevented LOOH formation in human LDLs (Fig. 3b) and reduced the amount and activity of MCP-1 (Fig. 3c, d) in conditioned medium. These results indicate that *PON1*$^{-/-}$ HDLs are unable to prevent LDL oxidation because they lack PON1.

Platelet-activating factor acetyl hydrolase (PAF-AH), another enzyme carried on HDL, can also destroy biologically active oxidized lipids *in vitro*[16]. Although the activity of PAF-AH was not reduced in *PON1* null mice (data not shown), this HDL was unable to destroy oxidized lipids, indicating that PAF-AH-containing HDLs are ineffective in the absence of PON1.

As PON1 seems to protect against lipid oxidation, we tested circulating lipoprotein particles for oxidized lipids. The amount of LOOH in each of the HDLs isolated from *PON1*$^{+/+}$, *PON1*$^{+/-}$ and *PON1*$^{-/-}$ mice was 6.2 ± 0.5, 7.8 ± 0.4 and 8.2 ± 0.2 μg linoleic acid equivalent per mg protein, respectively (*PON1*$^{+/+}$ versus *PON1*$^{+/-}$, $P = 0.01$; *PON1*$^{+/+}$ versus *PON1*$^{-/-}$, $P = 0.003$). When added by themselves to co-cultured cells, *PON1*$^{-/-}$ HDLs (without LDLs) stimulated formation of LOOH (Fig. 3b), and MCP-1 levels (Fig. 3c) and chemotactic activity (Fig. 3d) in conditioned medium, whereas *PON1*$^{+/+}$ HDLs did not (Fig. 3b–d).

Circulating LDLs carry large amounts of antioxidants and previous studies have found no evidence of oxidation. We did not find significant levels of LOOH in freshly isolated LDLs from any *PON1* genotype either. But in co-culture, LDLs from *PON1* null mice stimulated more monocyte transmigration than did LDLs from wild-type (3.3-fold increase; $P < 0.0001$; Fig. 3e). Our results suggest that LDLs from *PON1* null mice are altered in some way that increases their ability to generate oxidized lipids, although the nature of this alteration is unclear.

Our results and previous human genetic epidemiological studies[8] indicate that reduced PON1 may support the development of atherosclerosis. We therefore examined atherosclerotic lesions in the aortae of *PON1* wild-type, heterozygous and knockout mice fed a high-fat, high-cholesterol diet for 15 weeks. To reduce any variation due to genetic heterogeneity, we backcrossed the *PON1* null allele for three generations of mice onto the atherosclerosis-susceptible C57BL/6J strain. In mice fed a high-fat diet, the levels of VLDLs/LDLs increased and HDLs and PON1 levels were reduced compared with those in mice fed a chow diet (Table 1). The amounts of VLDLs/LDLs and HDLs, as well as of other plasma lipids, were the same in *PON1*$^{+/+}$ and *PON1*$^{-/-}$ mice (Table 1), but the *PON1*$^{-/-}$ mice had significantly larger aortic atherosclerotic lesions (mean lesion area: 13,530 ± 1,240 μm^2 per section) than *PON1*$^{+/+}$ and *PON1*$^{+/-}$ mice (7,610 ± 1,290 μm^2 per section) ($P < 0.01$) (Fig. 3f). We examined several *PON1*$^{-/-}$ mice maintained on a chow diet and none showed any evidence of fatty streak lesions, indicating that hyperlipidaemia is required for the development of lesions in these mice (data not shown).

Our results show that *PON1* null mice are severely compromised with respect to the two processes of inactivating organophosphate poisons and of destroying oxidized lipids. These processes are important in that they determine the response to insecticide poisoning and the susceptibility to atherosclerosis or other inflammatory diseases. □

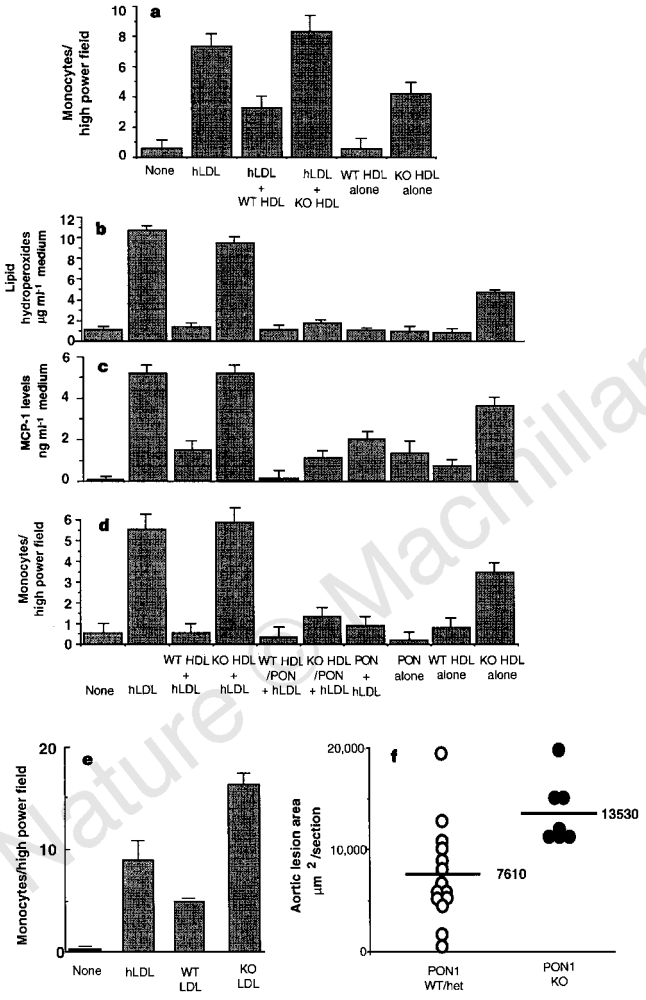

Figure 3 *PON1* null mice are susceptible to lipoprotein oxidation and atherosclerosis. **a**–**d**, HDL from *PON1* null mice fails to protect against LDL oxidation. Co-cultured cells were treated with various combinations of medium alone (none), human LDL (hLDL), wild-type mouse HDL (WT HDL), *PON1* null mouse HDL (KO HDL), HDL supplemented with purified PON1 (HDL/PON), or purified PON1 (PON). In **a**, co-cultures were treated as indicated for 8 h and conditioned medium was tested for monocyte chemotaxis. In **b**–**d**, co-cultures were pretreated with HDL, with or without supplements of PON1, for 18 h and then incubated with fresh medium, with or without hLDL. After incubation, the conditioned media were assayed for LOOH, MCP-1 or monocyte chemotaxis. **e**, Increased susceptibility of LDL from *PON1* null mice to oxidation. Co-cultured cells were treated with hLDL or with LDL from wild-type (WT LDL) or *PON1* null (KO LDL) mice, and conditioned media were assayed for monocyte chemotaxis. **f**, Increase in atherosclerosis in *PON1* null mice. Female *PON1*$^{+/+}$ or *PON1*$^{+/-}$ mice (PON1 WT/het, open circles) and *PON1*$^{-/-}$ mice (PON1 KO, filled circles) were fed on an atherogenic diet and the lesion area in the proximal aorta was determined. The average lesion sizes are indicated by bars.

Methods

Gene targeting. A gene targeting vector was constructed by first subcloning a 5.3-kb *Bgl*II DNA fragment containing exon 1 of the mouse *PON1* gene and flanking sequences into the *Bam*HI site of the pMC1TKpA vector. A neomycin-resistance gene expression cassette, MC1NEOpA (Stratagene), was then inserted into the *Nhe*I site of exon 1 of the *PON1* gene fragment. The targeting vector was linearized and electroporated into RW-4 embryonic stem (ES) cells derived from mouse strain 129/SvJ (Genome Systems). G418/gancyclovir-resistant clones were screened by Southern blot analysis and ES cells carrying the disrupted allele were microinjected into blastocysts of mouse strain C57BL/6J to produce chimaeric mice. Chimaeric mice were crossed with C57BL/6J and then intercrossed to produce mice homozygous for the *PON1* mutation.

Activity and immunoblotting of PON1. Plasma PON1 activity towards phenylacetate, paraoxon or chlorpyrifos oxon was determined as described[6,10]. For immunoblotting of PON1, plasma samples from *PON1* homozygous mutant mice and wild-type littermates were fractionated by SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with rabbit anti-mouse PON1 antiserum (1:1,000 dilution), washed, incubated with secondary antibody, and detected using electrochemiluminescence (Amersham).

Toxicity tests and acetylcholinesterase assays. $PON1^{+/+}$ and $PON1^{-/-}$ mice were treated with various doses of chlorpyrifos oxon or chlorpyrifos dissolved in 20 μl acetone by dermal exposure[10]. Four hours after treatment, mice were killed and their brains and diaphragms collected for assay of acetylcholinesterase activity. For mice that died within 4 h of treatment, tissues were collected immediately after death. AChE activity was assayed as described[9], using acetylthiocholine (ATC) as substrate and 5,5′-dithio-bis(nitrobenzoic acid) (DTNB) as chromogen. AChE activity was expressed as units per g of wet tissue (where one unit is the number of μmol ATC hydrolysed per min).

Plasma lipoproteins. Plasma lipids were determined by enzymatic colorimetric assay[17]. LDL and HDL density fractions were isolated from aliquots of pooled plasma from mice of both sexes or from human plasma by ultracentrifugation as described[15]. Lipid hydroperoxides in lipoproteins were measured as described[15].

Co-cultures. Co-culturing of human aortic endothelial cells and smooth muscle cells was done as described[14]; LDLs were used at a concentration of 350 μg protein ml^{-1}, HDLs at 500 μg protein ml^{-1}, and purified paraoxonase at 4 μg ml^{-1}. When co-cultures were pretreated with HDL or PON1, LOOH levels were determined after 8 h incubation with fresh medium, with or without human LDL; MCP-1 and monocyte chemotaxis were measured after incubation with fresh medium alone for an additional 18 h. MCP-1 was determined by ELISA (Antigenix America) and chemotaxis was assayed, as described[14]. For supplementation experiments, murine HDL at 500 μg ml^{-1} was incubated with purified human paraoxonase at 4 μg ml^{-1} at 37 °C with gentle shaking for 4 h. Unbound paraoxonase was removed by filtering through a 100K M_r-cutoff filter (Amicon). The activity of paraoxonase associated with HDL was determined before and after supplementation; HDLs were then used for co-culture experiments.

Diets and atheromatous-lesion analysis. Mice were fed on either a 6% fat chow diet or, for the atherosclerosis studies, an atherogenic diet containing 15.75% fat, 1.25% cholesterol and 0.5% sodium cholate (Teklad) for 15 weeks[17]. Quantification of aortic lesions has been described[17].

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Combined effects of angiostatin and ionizing radiation in antitumour therapy

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Angiogenesis, the formation of new capillaries from pre-existing vessels, is essential for tumour progression[1–5]. Angiostatin, a proteolytic fragment of plasminogen[6] that was first isolated from the serum and urine of tumour-bearing mice[7], inhibits angiogenesis and thereby growth of primary[8] and metastatic[7,9,10] tumours. Radiotherapy is important in the treatment of many human cancers, but is often unsuccessful because of tumour cell radiation resistance[11,12]. Here we combine radiation with angiostatin to target tumour vasculature that is genetically stable and therefore less likely to develop resistance[13–15]. The results show an antitumour interaction between ionizing radiation and angiostatin for four distinct tumour types, at doses of radiation that are used in radiotherapy. The combination produced no increase in toxicity towards normal tissue. *In vitro* studies show that radiation and angiostatin have combined cytotoxic effects on endothelial cells, but not tumour cells. *In vivo* studies show that these agents, in combination, target the tumour vasculature. Our results provide support for combining ionizing radiation with angiostatin to improve tumour eradication without increasing deleterious effects.

To assess the effects of human angiostatin on primary tumour growth, we treated mice with murine Lewis lung carcinoma (LLC)