Oxidative and nitrative stress caused by subcutaneous implantation of a foreign body accelerates sarcoma development in Trp53<sup>+/−</sup> mice

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Chronic inflammation is a recognized risk factor for human cancer at various sites because of persistent oxidative and nitrative tissue damage. Trp53<sup>+/−</sup> mice show the predisposition to tumor development, such as sarcomas and lymphomas, compared with Trp53<sup>+/+</sup> mice. We investigated the effects of chronic inflammation, especially oxidative and nitrative stress, induced by subcutaneous implantation of a plastic plate (10 × 5 × 1 mm) as a foreign body on tumorigenesis in Trp53<sup>+/−</sup> and Trp53<sup>+/+</sup> mice. The plastic plates were implanted at the age of about 11 weeks. Thirty out of 38 Trp53<sup>+/+</sup> mice (79%) developed sarcomas around the implant (mean time of tumor appearance was 45.8 ± 12.0 weeks of age), whereas only one of 10 Trp53<sup>+/−</sup> mice with an implant (10%) developed a tumor, at 56 weeks. No sarcomas developed at a sham-operation site. Two of 10 Trp53<sup>+/−</sup> mice with no implant (20%) also developed three sarcomas spontaneously at 77, 81 and 84 weeks. Increased immunostaining for markers of oxidative and nitrative stress (8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-nitroguanine and 3-nitrotyrosine) and expression of inducible nitric oxide synthase in tumor cells and inflammatory cells were detected in implant-induced sarcomas compared with spontaneous sarcomas in Trp53<sup>+/−</sup> mice. Furthermore, p53 loss of heterozygosity was observed in 26 out of 29 implant-induced sarcomas (90%). These results indicate that implanted foreign bodies significantly enhanced sarcoma development in Trp53<sup>+/−</sup> mice, and this may be associated with increased oxidative and nitrative stress. Loss of the remaining wild-type p53 allele and loss of p53 function appears to be, at least in part, underlying molecular mechanisms during the development of sarcomas at the implantation site in Trp53<sup>+/−</sup> mice. Such implant-induced sarcoma development in Trp53<sup>+/−</sup> mice could be useful for studying molecular mechanisms and developing new strategies for chemoprevention in human carcinogenesis induced by chronic inflammation and/or foreign bodies.

Introduction

Chronic inflammation induced by biological, chemical and physical factors has long been implicated in human carcinogenesis at various sites (1–4). In inflamed tissues, many types of inflammatory mediators, including reactive oxygen and nitrogen species, cytokines, growth factors and chemokines, are generated by both inflammatory cells and inflamed epithelial and stromal cells, and form complex pathways in inflammatory processes (1,2). However, excess amounts of reactive oxygen and nitrogen species produced by inflammatory cells may cause oxidative and nitrative damage in DNA and proteins in inflamed tissues, thus contributing to the initiation and/or promotion of cancer (3,4). Inducible nitric oxide synthase (iNOS) expressed in inflammatory cells may generate a variety of reactive oxygen and nitrogen species, including nitric oxide (NO<sub>•</sub>), superoxide (O<sub>2</sub>•<sup>−</sup>), nitroxylin anion (NO<sup>•</sup>) and peroxynitrite (ONOO<sup>−</sup>), which is formed by reaction of NO<sup>•</sup> with O<sub>2</sub>•<sup>−</sup> (3–5). These species can cause severe oxidative and nitrative stress (3–6). 8-Nitroguanine (8-nitroG) has been detected immunohistochemically in inflamed tissues such as the lung of mice infected with influenza virus (7), the bile duct of hamsters infected with liver fluke (8) and the gastric mucosa of humans infected with Helicobacter pylori (9), and hence has been considered as a marker for nitrative nucleic acid damage (5,6). In vitro experiments suggest that its formation in DNA may induce G:C to T:A transversions, which are among the most common mutations in oncogenes and tumor-suppressor genes in various human cancers, possibly through formation of depurination-dependent abasic sites (10) and/or direct mispairing with adenine (11). 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG) has been measured as a marker for oxidative DNA damage and has been shown to cause G:C to T:A transversions (3–5,12). 3-Nitrotyrosine (NTYR) is a marker for nitrosatively damaged proteins, which are formed by nitration of tyrosine residues in proteins with reactive nitrogen species (5). More than 40 proteins containing NTYR have been identified in tissues associated with inflammatory conditions in vivo (13). Accumulation of these markers for oxidative and nitrative stress may be involved in inflammation-associated carcinogenesis.

Heterozygous p53-deficient (Trp53<sup>+/−</sup>) mice with only one functional allele in the p53 gene develop spontaneous sarcomas (14,15), like patients with Li-Fraumeni syndrome carrying an inherited p53 mutation, who develop sarcomas at a very early age (16). Furthermore, some carcinogens, such as dimethylnitrosamine and N-methylnitrosourea, induce
sarcoma development in Trp53<sup>+/−</sup> mice within shorter periods than in p53 wild-type (Trp53<sup>+/+</sup>) mice (15,17). Although the molecular mechanism of sarcoma development in Trp53<sup>+/−</sup> mice remains largely unclear, loss of heterozygosity (LOH) at the Trp53 locus has been reported to be more frequent in carcinogen-induced sarcomas (18) than spontaneous tumors including sarcomas (19), suggesting that the induction of p53 LOH may provide a strong advantage toward sarcoma development in Trp53<sup>+/−</sup> mice.

Implantation of a foreign body has been shown to cause sarcoma development around the implant in a strain- and gender-dependent manner (20). Inflammatory conditions around a foreign body, such as infiltration of phagocytes, fibrous connective tissues and active cellular proliferation, are recognized as major etiological factors in sarcoma development (21), although the exact molecular mechanism remains unknown. In the present study, we investigated the effects of chronic inflammation, especially oxidative and nitrative stress, induced by subcutaneous implantation of plastic plates on tumorigenesis in Trp53<sup>+/−</sup> and Trp53<sup>+/+</sup> mice. We also investigated p53 LOH in implant-induced tumors from Trp53<sup>+/−</sup> mice.

Materials and methods

Animals

Trp53<sup>+/−</sup> mice (C57BL/6J-129Sv) established by Donehower et al. (14) were maintained in a C57BL/6 background, as reported previously (22). To obtain male Trp53<sup>+/−</sup> and Trp53<sup>+/+</sup> mice, Trp53<sup>+/−</sup> mice obtained by breeding Trp53<sup>−/−</sup> mice with Trp53<sup>+/+</sup> mice (C57BL6) were intercrossed. Genotyping at the Trp53 locus was performed for all mice used in this study, as reported previously (22). Mice were housed in pathogen-free conditions. This study was approved by the IARC Animal Use and Care Committee.

Preparation and implantation of plastic plates

Plastic plates (polystyrene, 10 × 5 × 1 mm) were prepared from 100 mm culture dishes (Falcon, 353003) and sterilized by UV irradiation before implantation. Mice were anesthetized by intraperitoneal injection of 2,2,2-trifluoroethanol (300 mg/kg body wt) (Fluka, Buchs, Switzerland), and the plastic plates were subcutaneously implanted on the right side of the back in the acute inflammatory phase. These tissues were examined histologically at the acute inflammatory phase. These tissues were examined histologically and immunohistochemically.

Immunohistochemistry

Tumors and subcutaneous tissues were fixed in 10% neutral buffered formalin. Paraffin-embedded sections (4 μm) were prepared for hematoxylin and eosin staining and immunohistochemical examination. Presence of 8-nitroG and 8-oxodG was determined by double immunofluorescence labeling techniques, as reported previously (8,24). After deparaffinization and rehydration, tissue sections were incubated with rabbit polyclonal anti-8-nitroG and 8-oxodG was determined by double immunofluorescence labeling with 8-nitroG. Monoclonal anti-iNOS antibody (1:1000; Sigma, St Louis, MO) was used as reported previously (24). For NTYR staining, antigen activation was performed by microwave irradiation at 750 W for 20 min in 10 mM citrate buffer (pH 6.0). The tissue sections were then incubated with rabbit polyclonal anti-3-nitrotyrosine antibody (2 μg/ml; Upstate Biotechnology, Lake Placid, NY) overnight and goat horseradish peroxidase-labeled polymer against rabbit IgG (Dako, Carpinteria, CA). Signals were developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Dako). The nuclei were counterstained with hematoxylin.

To quantify the immunoreactivity for 8-nitroG, 8-oxodG and iNOS in tumors, signals were developed by DAB solution without the counterstaining of nuclei following the same protocol described above in three implant-induced and three spontaneous sarcomas. The photographs of immunostained sections were obtained under ×400 magnification in five randomly selected fields in each tumor. We made all the photographs binary and calculated the integrated densities of immunoreactive cells for 8-nitroG, 8-oxodG and iNOS in tumors by NIH Image software (version 1.63) on a Macintosh computer.

Analysis of LOH at the Trp53 locus

Genomic DNA was isolated from frozen tissue of tumors and livers from the same mice using a Qiagen (Chatsworth, CA) kit. Trp53<sup>+/−</sup> mice used in this study had one mutated allele, in which intron 4 and exon 5 were partially deleted (14), and one wild-type allele in the same mice to examine the effect on tumorigenesis of acute inflammation induced by skin incision. The mean ages at implantation in implant-induced tumors from wild-type (+/+) and deletion (−/−) mice, respectively. A sham-operation was performed in tumorigenesis of acute inflammation

Statistical analysis

Statistical significance of differences in tumor incidence was determined by Fisher’s exact test. The difference in curves of tumor incidence with time was determined by two-tailed Mann–Whitney U-test. P < 0.05 was considered significant.

Results

Subcutaneous implantation of plastic plates accelerated sarcoma development in Trp53<sup>+/−</sup> mice

Thirty out of 38 Trp53<sup>+/−</sup> mice (79%) developed a subcutaneous tumor around an implanted plastic plate, with a
Fig. 2. Sarcoma developed in Trp53+/− mice implanted with plastic plates. Trp53+/− mice developed tumors at the implantation site (black arrow), but not at the sham-operation site (white arrow) (A). The plastic plates were present within tumor tissues. PP indicates empty space where the plastic plate was present (B). Tumors at the implantation sites were malignant fibrous histiocytomas (C–E), with fibrous (D) or pleomorphic (E) areas. Original magnification: B, ×40; C, D, E, ×400.

mean time of tumor appearance of 45.8 ± 12.0 (mean ± SD) weeks (range: 29–76 weeks) of age, whereas only one of 10 Trp53+/− mice (10%) with an implant developed a subcutaneous tumor at the site of implantation, at 56 weeks of age. The tumor incidence in Trp53+/− mice with an implant was significantly greater than that in Trp53+/+ mice with an implant (P < 0.0001) (Figures 1 and 2A). No tumor was observed at the site of sham-operation in any of the mice examined (Figure 2A). In all tumors that developed at an implantation site, plastic plates were present in the center or the lateral parts of the tumor tissue (Figure 2B). Two of 10 Trp53+/− mice (20%) without an implant developed a subcutaneous tumor spontaneously: one mouse developed a tumor at 77 weeks of age and another mouse developed two sarcomas at 81 and 84 weeks.

Table I. Comparison of tumor incidence and appearance period between implanted and untreated Trp53+/− mice

<table>
<thead>
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<th>Treatment</th>
<th>Tumor incidence (%)</th>
<th>Tumor appearance (weeks)</th>
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<tr>
<td>Implanted</td>
<td>30/38 (79)a</td>
<td>45.8 ± 12.0b</td>
</tr>
<tr>
<td>Untreated</td>
<td>2/10 (20)</td>
<td>77, 81, 84d</td>
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aP < 0.0001 compared with untreated Trp53+/− mice.
bP < 0.0001 compared with untreated Trp53−/− mice.

Among eight Trp53+/− mice with no tumor at the implantation site, one died of malignant lymphoma, infiltrating into the spleen and liver, and one died of sarcoma of the face. Another two mice died of unknown causes. The four remaining mice were still alive 70 weeks after implantation.

Increased oxidative and nitrative stress and iNOS expression in implant-induced sarcomas

Figure 3 shows the immunostaining of markers for oxidative and nitrative stress, such as 8-nitroG, 8-oxodG and NTYR, and iNOS expression in implant-induced and spontaneous sarcomas from Trp53+/− mice. Implant-induced sarcomas showed strong immunoreactivity for 8-nitroG and 8-oxodG in the nuclei and cytoplasm of tumor cells and infiltrating inflammatory cells (Figure 3A). 8-OxodG immunoreactivity was more diffuse than that of 8-nitroG. In contrast, spontaneous sarcomas showed weak accumulation of these markers. The average of integrated density for immunoreactive cells with 8-nitroG in three implant-induced and three spontaneous sarcomas was 3798 ± 1312 (mean ± SD) and 169 ± 136, respectively. The mean integrated density of 8-oxodG immunoreactivity was 2942 ± 817 and 193 ± 127 in implant-induced and spontaneous sarcomas, respectively. Implant-induced sarcomas showed significantly greater immunoreactivity of 8-nitroG and 8-oxodG than spontaneous sarcomas (P < 0.001) (Figure 4). NTYR immunoreactivity was clearly seen in the cytoplasm of tumor cells and inflammatory cells, such as multinuclear giant cells, in implant-induced sarcomas, whereas its immunoreactivity was weak in spontaneous sarcomas (Figure 3B). Expression of iNOS was co-localized with 8-nitroG immunoreactivity in inflammatory cells and tumor cells in implant-induced sarcomas (Figure 3C). In contrast, spontaneous sarcomas showed weak iNOS immunoreactivity in cells of both types. The mean integrated density of iNOS immunoreactivity was 2388 ± 723 and 409 ± 344 in implant-induced and spontaneous sarcomas, respectively. In implant-induced sarcomas, showed significantly greater iNOS immunoreactivity than spontaneous sarcomas (P < 0.001) (Figure 4). Similarly to Trp53+/− mice, implant-induced sarcomas from one Trp53+/+ mouse showed strong accumulation of all these markers (data not shown).

Oxidative and nitrative stress-induced sarcoma in Trp53+/− mice

Figure 5 shows the immunostaining of markers for oxidative and nitrative stress and iNOS expression in subcutaneous tissues around implants on Day 14 after implantation in Trp53+/+ mice. The implanted plastic plates were covered by fibrous tissue capsules with the accumulation of inflammatory cells in the surrounding stromal tissues. Immunoreactivity of 8-nitroG and 8-oxodG was co-localized in the nuclei and cytoplasm of infiltrating inflammatory cells, stromal cells and fibroblasts. In contrast, iNOS was expressed in the cytoplasm of inflammatory cells and stromal cells co-localizing with 8-nitroG formation, but its immunoreactivity was weak in fibrous tissues. Subcutaneous tissues around plastic plates on Day 14 after implantation in Trp53+/− mice also showed similar patterns of immunostaining of these.
markers (data not shown). In contrast, on Day 7 after implantation, immunoreactivity of all markers was weaker than that on Day 14 at the implantation sites in both types of mouse (data not shown). No immunoreactivity without primary antibody for 8-nitroG (m) or 8-oxodG (n) was observed. (B) Strong accumulation of NTYR was observed in tumor cells and inflammatory cells including multinuclear giant cells (black arrows) in Imp-S (a) compared with Spo-S (b). (C) Imp-S showed strong iNOS expression co-localized with 8-nitroG immunoreactivity in tumor cells and inflammatory cells (a–d). Spo-S showed weak iNOS expression and 8-nitroG formation (e–h). Original magnification: A—a, b, c, d, ×40; e, f, g, h, i, j, k, l, m, n, ×200; B—a, b, ×400; C—a, b, c, d, e, f, g, h, ×400.

Analysis of LOH at the Trp53 locus in implant-induced sarcomas from Trp53⁺⁻ mice

Figure 6 shows representative results showing both presence and absence of LOH at the Trp53 locus in implant-induced sarcomas from Trp53⁺⁻ mice. Normal liver from each mouse showed two strong bands corresponding to exon 5 and 7.
Imp-S showed significantly (three implant-induced (Imp-S) and three spontaneous (Spo-S) sarcomas. 8-nitroG, 8-oxodG and iNOS were determined by NIH Image software in densities of five randomly selected fields in the sections immunostained with immunostained with 8-nitroG and developed by DAB (a, b) and binary photographs.

Fig. 4. Quantification of immunoreactive cells for 8-nitroG, 8-oxodG and iNOS in implant-induced and spontaneous sarcomas. (A) The integrated densities of five randomly selected fields in the sections immunostained with 8-nitroG, 8-oxodG and iNOS were determined by NIH Image software in three implant-induced (Imp-S) and three spontaneous (Spo-S) sarcomas. Imp-S showed significantly (P < 0.001) greater immunoreactivities for all markers than Spo-S. (B) Representative photographs of sections immunostained with 8-nitroG and developed by DAB (a, b) and binary ones (c, d) in Imp-S and Spo-S. Original magnification: a, b, c, d, ×200. The integrated densities for binary ones were shown under the photographs.

whereas sarcomas from three Trp53+/− mice (Nos. 386, 398 and 409) exhibited a much weaker band for exon 5 than for exon 7, indicating the presence of p53 LOH in these sarcomas. In contrast, sarcomas from two other Trp53+/− mice (Nos. 374 and 439) showed two strong bands for exons 5 and 7 like normal liver, indicating a lack of p53 LOH. Twenty-six of 29 (90%) implant-induced sarcomas exhibited p53 LOH.

Discussion

In this study, we have clearly demonstrated that subcutaneous implantation of a foreign body in Trp53+/− mice increased the incidence of sarcoma developing around the implant and shortened the tumor latency, compared with the data for spontaneous sarcomas that developed in Trp53+/− mice without an implant. The incidence of sarcomas in Trp53+/− mice with an implant was also greater than that in similarly implanted Trp53+/− mice. No tumors developed at the sham-operation sites, suggesting that chronic inflammation induced by implantation, but not acute inflammation, may contribute to the rapid sarcoma development in Trp53+/− mice.

Although many cytotoxic and mutagenic agents have been shown to induce tumors at various sites in Trp53+/− mice more rapidly than Trp53+/− mice (27), this study has clearly demonstrated that chronic inflammation alone, without any carcinogen, enhances and accelerates tumorigenesis in Trp53+/− mice. Blanchard et al. (28) previously reported similar enhancements of sarcoma development by foreign bodies (glass and polypropylene microchips) in Trp53+/− mice. However, they observed their mice for only 26 weeks after implantation, and only 18 of 177 Trp53+/− mice (10%) developed sarcomas after implantation, while no tumors were induced in Trp53+/− mice with implants. In the present study, we observed animals for up to 70 weeks after implantation and found that 30 of 38 Trp53+/− mice (79%), but only one of 10 Trp53+/− mice (10%), developed sarcomas around implants. At 26 weeks after implantation, only 7 of the 38 Trp53+/− mice (18%) and none of the 10 Trp53+/− mice (0%) developed sarcomas, similar to the data reported by Blanchard et al. (28).

We observed increased accumulation of 8-nitroG, 8-oxodG and NTYR in implant-induced sarcomas compared with spontaneous sarcomas. Expression of iNOS was also increased in implant-induced sarcomas, suggesting the involvement of oxidative and nitrative stress induced by iNOS expression in sarcoma development. As has been reported previously (21), we observed that implantation of a foreign body induced the infiltration of inflammatory cells and the formation of a fibrous tissue capsule around the implant at the acute inflammatory phase (on Day 14). Inflammatory cells and surrounding stromal cells around the implant showed increased immunoreactivity for 8-nitroG and 8-oxodG, consistent with increased expression of iNOS. Fibroblasts forming the fibrous tissue capsule exhibited strong accumulation of 8-nitroG and 8-oxodG in spite of weak iNOS expression. Our results suggest that the infiltration of inflammatory cells around implanted foreign bodies may induce oxidative and nitrative stress in stromal and fibrous tissues through iNOS expression, resulting in sarcoma development. To address the role of iNOS on implant-induced sarcoma development, we will further investigate additional animal experiments using iNOS inhibitor or Trp53+/− mice disrupted in the iNOS gene.

As LOH at the Trp53 locus has been shown to be frequent in carcinogen-induced sarcomas in Trp53+/− mice (18), we examined p53 LOH in implant-induced sarcomas. Similar to carcinogen-induced sarcomas (18), we found frequent p53 LOH (90%) in implant-induced sarcomas. We also observed infrequent p53 LOH in spontaneous sarcomas (1 out of 3, 33%) (Our unpublished data). Recent studies suggest the involvement of homologous recombination in the induction of LOH in tumor-suppressor genes (29). Reactive oxygen and nitrogen species, such as H2O2 (30), NO (31) and ONOO− (32), have been reported to induce homologous recombination in human cells and Escherichia coli. Irradiation causing severe tissue damage has been shown to induce increased homologous recombination and genetic instability in normal tissues of Trp53-deficient mice (33,34). Thus, our results suggest that p53 LOH may be induced by excess reactive oxygen and nitrogen species, produced as part of the inflammatory response to the foreign body, leading to complete loss of p53 function in Trp53+/− mesenchymal cells around the implant. It will be a further research goal to clarify the association between oxidative and nitrative stress and the induction of p53 LOH.
Implant-induction of sarcoma in experimental animals has recently been shown to be useful for studying the mechanisms of initiation and promotion in sarcoma development, because preneoplastic lesions can be easily identified around foreign bodies (35). Furthermore, Okada et al. (23,36) previously reported that when poorly tumorigenic and non-metastatic mouse fibrosarcoma cells (QR-32) were co-implanted with foreign bodies such as plastic plates and gelatin sponge, the cells acquired a phenotype of highly malignant and metastatic capability. Sarcoma development associated with implants in \( Trp53^{+/+} \) mice has the further advantages of higher incidence and shorter tumor latency compared with those in \( Trp53^{+/+} \) mice. This animal model would therefore be useful in studying the molecular mechanisms underlying the effects of chronic inflammation on cancer initiation, promotion and progression.

We believe that the present animal study will contribute to understanding inflammation-related carcinogenesis in humans. Mallery et al. (37) has recently shown the possible role of oxidative and nitrative stress in the pathogenesis of AIDS-related Kaposi’s sarcoma, which develops through a persistent inflammatory condition after profound immunosuppression caused by HIV infection. Furthermore, in foreign body-induced carcinogenesis in humans, asbestos fibers are well known to induce malignant mesotheliomas after chronic inhalation. Intraperitoneal injection of asbestos fibers has been also shown to accelerate malignant mesotheliomas in \( Trp53^{+/+} \) mice (38). We have demonstrated that 8-nitroG is formed specifically at the sites of carcinogenesis under various inflammatory conditions in humans and experimental animals (39). 8-NitroG was apparently formed in gastric gland epithelial cells of patients with \( H.pylori \) infection (9) and in hepatocytes of patients with chronic hepatitis C (40). Furthermore, 8-nitroG formation was observed in oral epithelium of patients with oral precancerous conditions (41,42). Thus, these findings suggest that \( Trp53^{+/+} \) mice implanted with foreign bodies at various organs may be useful as a clinically relevant model for various inflammation-related

\[ \text{LOH}^{(+)} \]
\[ \text{LOH}^{(-)} \]

Fig. 6. Analysis of LOH at the \( Trp53 \) locus in implant-induced sarcomas from \( Trp53^{+/+} \) mice. Normal liver (L) from all mice showed the two clear bands for exon 5 (311 bp) and exon 7 (278 bp). Tumors (T) from three mice (Nos. 386, 398 and 409) showed a very weak band for exon 5, indicating LOH. Tumors from two other mice (Nos. 374 and 439) showed a clear band for exon 5, indicating no LOH. M, marker; N, negative control.

Fig. 5. Immunohistochemistry of nitrative and oxidative stress markers and iNOS in subcutaneous tissues around implants at the acute inflammatory phase in \( Trp53^{+/+} \) mice. On Day 14 after implantation, the plastic plate (PP) was covered by fibrous tissue capsule (F) with accumulation of inflammatory cells (a, e, i). 8-NitroG and 8-oxodG were accumulated in inflammatory cells, fibroblasts and stromal cells around the PP (b–d, f, j). In contrast, iNOS expression was observed in infiltrating inflammatory cells and surrounding stromal cells (g, h, k, l). Original magnification: a, b, c, d, e, f, g, h, ×200; i, j, k, l, ×400.
human cancers and may further provide us the opportunity to develop cancer-preventive strategies.

In conclusion, subcutaneous implantation of plastic plates accelerated sarcoma development in Tpr53−/− mice. Oxidative and nitrative damage caused by inflammatory cells infiltrating around the implant may be implicated in sarcoma development probably through the induction of p53 LOH.

Our results suggest that chronic inflammation may be a risk factor for development of a variety of cancers, including sarcomas.

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