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MexTAg mice exposed to asbestos develop cancer that faithfully replicates key features of the pathogenesis of human mesothelioma

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ABSTRACT

Animal models provide an important tool for investigating the biology of cancer and for testing the efficacy of novel treatments. Here we describe several aspects of the transgenic MexTAg mouse that develops asbestos-induced mesothelioma. Targeted expression of the TAg transgene causes mesothelioma to develop more rapidly after asbestos exposure in wild-type mice with 100% incidence compared to 30% incidence in wild-type mice. MexTAg mice do not develop spontaneous mesothelioma and exhibit a very low incidence of other tumours. Here we show that TAg does not affect the aggressiveness or rate of progression of the mesotheliomas, suggesting that the oncogene alters only the rate at which disease is initiated. The instillation of an alternative inflammatory agent, thioglycollate, did not induce mesotheliomas, demonstrating acute inflammation is not sufficient for tumour development in MexTAg mice. We found that neither the age of a mouse at the time of exposure nor its gender were prognostic factors. MexTAg mice with mesotheliomas respond to treatment with a cytotoxic drug in a similar way to that of patients with mesothelioma, demonstrating the validity of the model. We also show that long-term treatment with a COX-2 inhibitor prior to the development of disease does not have a survival benefit, suggesting that this is not a useful cancer prevention therapy for asbestos-exposed individuals. The model is robust and suitable for testing a wide variety of protocols and a range of translational studies.

1. Introduction

Asbestos is the principal cause of mesothelioma and significant exposure is recorded in up to 80% of cases.[1] The disease is characterised by a long latency period of 25–60 years between exposure and clinical presentation. Untreated, the median survival is less than 12 months from diagnosis.[2,3] Once disease develops, treatment options such as chemotherapy prolong survival but rarely result in cures.[4] The period between asbestos exposure and development of disease may be a window of opportunity for chemoprevention or dietary interventions to delay or reduce the risk of mesothelioma development. This strategy has succeeded in preventing cancer in some other groups, such as women at high risk of breast cancer.[5] However, as mesothelioma is relatively rare, and there are as yet no biologically plausible druggable targets for chemoprevention, a

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translatable pre-clinical model for screening potential preventive therapies is needed.

Animal models of cancer provide the groundwork for clinical trials of novel therapies, providing important information on toxicity, dose, scheduling and efficacy. There are three main types of animal model that are widely used in this regard: the induction of cancer using a carcinogen; the transplantation of a cancer cell line into syngeneic or immunocompromised mice; and transgenic mice with a genetic predisposition to a cancer of interest.[6] Variations in these strategies include the use of carcinogens in genetically modified animals to accelerate the course of disease or increase the penetrance of the disease. Each system has advantages and disadvantages so that ultimately the selection of model depends upon the question that is being asked. Subcutaneous transplantation has been extensively used to study mesothelioma, but despite predictable tumour development, rapid growth, and ease of access for measurement and interventions, it is not suitable for testing chemopreventative strategies following carcinogen exposure.

We recently generated MexTAg transgenic mice that express SV40 large T antigen specifically in mesothelial cells by use of the cell-type-specific mesothelin promoter.[7,8] There are four separate MexTAg mouse lines: 299h (high), 304i (intermediate high), 270i (intermediate low) and 266s (single) which have 100, 32, 15 and 1 copies of the transgene, respectively. A strength of this model is the requirement for asbestos exposure to induce mesotheliomas. MexTag animals that are not asbestos exposed do not develop the disease. Whilst only 20–30% of wild-type mice develop mesotheliomas after intraperitoneal asbestos inoculation, this rises to 100% in the MexTAg mice, making them a suitable and efficient system for pre-clinical research.[7] Disease develops rapidly in MexTAg mice, occurring from 20 to 40 weeks after exposure, compared to 50–100 weeks in unmodified mice. Thus, the mesotheliomas that develop after asbestos exposure in MexTAg mice approximate human disease in terms of tumour location, induction by the same carcinogen, and a comparable latency period between asbestos exposure and disease development in terms of murine lifespan.

These features make MexTAg mice a suitable system for addressing questions regarding the development of mesothelioma prior to clinical presentation, the potential carcinogenicity of other asbestos-like materials, the effect of age and gender on disease development and the potential of dietary supplementation or pharmacological interventions to delay or prevent mesothelioma. Here, we demonstrate how the model can be utilised in these settings, with outcomes that are consistent with our current understanding of the pathogenesis of human mesothelioma. We propose that the MexTag model of asbestos-induced mesothelioma is an ideal system for testing novel therapies and cancer prevention agents.

2. Materials and methods

2.1. Transgenic mice

MexTAg transgenic mice were generated by insertion of a 2148-bp fragment of the SV40 TAg open reading frame cloned downstream of 1850 bp of the mesothelin promoter as described previously.[7] Founders with four different copy numbers of the transgene have been maintained as separate mouse lines: MexTAg 299h (100 copies), 304i (32 copies), 270i (15 copies) and 266s (single copy). Mice were bred as heterozygotes and selected for experiment in groups which were matched as closely as possible for age and gender balance, unless otherwise stated.

2.2. Asbestos-induced mesothelioma

Asbestos fibres (IUCC reference sample of Wittenoom Gorge crocidolite) were suspended in PBS at a concentration of 6 mg/ml, then autoclaved and passaged through a 23 gauge needle several times. Groups of mice (C57/Bl 6J wild-type and MexTAg transgenic) were injected in the peritoneum (i.p.) with two doses of 3 mg asbestos, 1 month apart; survival was taken from the date of the first asbestos injection. All experiments were repeated. Mice were monitored and euthanised according to ethically approved guidelines pertaining to animal health including signs of sickness, distress or loss of condition. All experiments had UWA animal ethics approval and were carried out according to these protocols.

2.3. Immunohistochemistry

Tissue samples from the pleura, heart, lung, liver, diaphragm, kidney, peritoneal wall, testis, intestine, spleen and pancreas were fixed in 4% paraformaldehyde v/v and preserved in ethanol until preparation into paraffin blocks by standard procedures. Five micrometre sections were cut from paraffin blocks, dewaxed in xylene and rehydrated in a graded series of alcohols. The SV40 pAb 101 antibody was applied for 1 h at 1:100 dilution at room temperature and detected using an anti-mouse HRP conjugate using the SuperPicTure Polymer detection kit (Zymed laboratories, Inc., San Francisco, CA) according to manufacturer's instructions.

2.4. Thioglycollate inoculation

Thioglycollate was prepared as a 3% w/v solution in water and allowed to oxidise for 4 weeks prior to use. Mice were injected in the peritoneum (i.p.) with 0.5 ml of the 3% thioglycollate solution and monitored for any signs of disease development for up to 45 weeks. At 45 weeks mice were euthanised and an autopsy was carried out to assess the presence of tumours or other signs of disease particularly those previously described in asbestos-treated mice.[7]

2.5. Peritoneal wash and differential cell counts

Mice were euthanised at 48 h, 1 week, 4 weeks and 16 weeks after a single i.p. injection of asbestos and injected intraperitoneally with 4 ml of sterile saline (Baxter). The whole peritoneal wash was then drawn out with a 21G needle. Cells were centrifuged at 1200 rpm for 10 min at 4 °C. Supernatant was removed and cells were resuspended in media containing 10% foetal calf serum v/v (Invitrogen, Life Sciences), 20 mM HEPES (Sigma), 100 U/ml benzylpenicillin (CSL), 50 μg/ml gentamicin (Pfizer) and 0.05 mM 2-mercaptoethanol (Sigma).

Cells were analysed for differential counts on the Hemavet 950FS (Drew Scientific) Coulter counter.

2.6. Chemotherapy

Groups of 10 MexTAg 299h mice were injected i.p. with 120 mg/kg gemcitabine twice weekly, in a cycle of 3 followed by 4 d apart. Control mice were injected with 100 μl phosphate-buffered saline (PBS). Treatment started 16 weeks after the first asbestos injection and continued until mesothelioma had developed. This experiment was repeated.

2.7. Celecoxib diet

Celecoxib was purchased from LKT laboratories, Inc., St. Paul, MN Sigma and added at a concentration of 1.5 g/kg to a standard mouse diet AIN93 (Specialty Feeds, Western Australia), containing all required nutrients. The diet was irradiated and provided *ad libitum* 1 d prior to the first asbestos injection i.p. for the duration of the experiment.

2.8. Serum celecoxib levels

Tail vein blood samples were taken at selected time points following provision of the test diet, allowed to clot and centrifuged to enable collection of serum samples which were immediately frozen and analysed collectively a few weeks later. Serum celecoxib levels were measured by Liquid Chromatography–Electrospray Ionisation Tandem Mass Spectrometry at the Clinical Pharmacology and Toxicology Laboratory, Path West, Nedlands, Perth, Western Australia.

2.9. Statistical analyses

Kaplan–Meier survival curves were analysed by log rank test for survival, with >95% confidence intervals (CI). Correlations

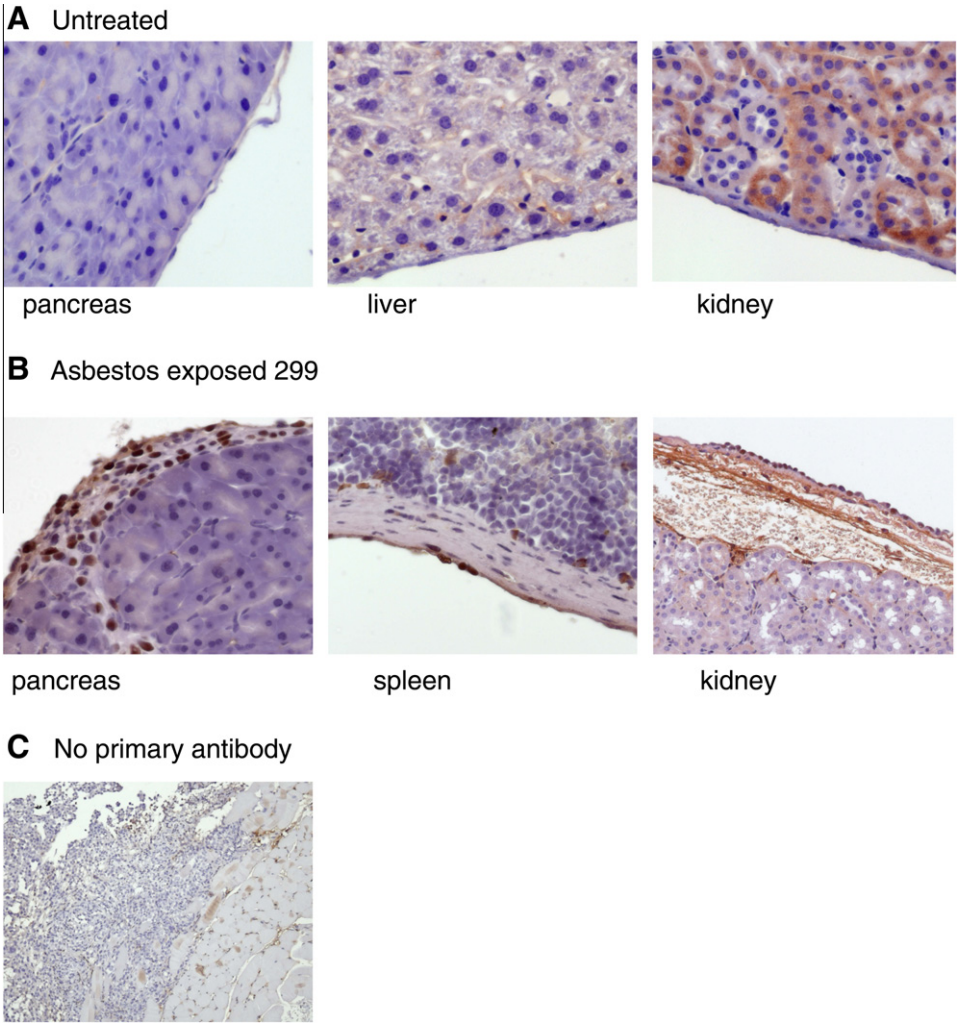

Fig. 1 – MexTAg mice do not express detectable TAg protein prior to asbestos exposure. Tissue samples, as indicated, were dissected from untreated (panel A) and asbestos-exposed (panel B) MexTAg 299h mice and immunohistochemistry (IHC) was performed using the pAb 101 anti-SV40 TAg antibody. TAg-positive cells are identified by brown nuclear staining. IHC control (C) is a section of mesothelioma attached to the peritoneum of an asbestos-exposed MexTAg 299h mouse without pAb101 antibody.

were analysed by Pearson's test for correlation with 95% CI. The one-way Anova test for variance was used to analyse data from three or more test groups. The non-parametric, unpaired, two-tailed t-test was used to compare data from two test groups.

3. Results

3.1. TAg protein is expressed in MexTAg mice only after exposure to asbestos

In MexTAg mice, transgene expression is directed to the mesothelial cells that line the surface of the peritoneal and pleural cavities and the organs within them.[7] However, MexTAg mice do not develop mesothelioma in the absence of a tumour promoter. Because we know that TAg is a powerful oncogene and we wanted to better understand why untreated animals remained tumour free, we used immunohistochemistry to examine TAg expression in tissues from untreated or asbestos-exposed MexTAg mice. No TAg was detected in samples that were not directly exposed to asbestos (Fig. 1A). In contrast, TAg was highly expressed in mesothelial cells, reactive mesothelial cells and mesothelioma cells of tissues that had been exposed to asbestos (Fig. 1B).

3.2. Thioglycollate and asbestos trigger an inflammatory reaction in the peritoneum

Thioglycollate is an irritant that causes an inflammatory response similar to that seen after asbestos injection.[9] In order to examine whether the development, characteristics, and persistence of a peritoneal infiltrate differed between asbestos and a non-carcinogenic irritant, we assessed the cellular composition of infiltrates induced by asbestos and thioglycollate in the MexTAg 299h mice, at 48 h and then at 1, 4 and 16 weeks after injection.

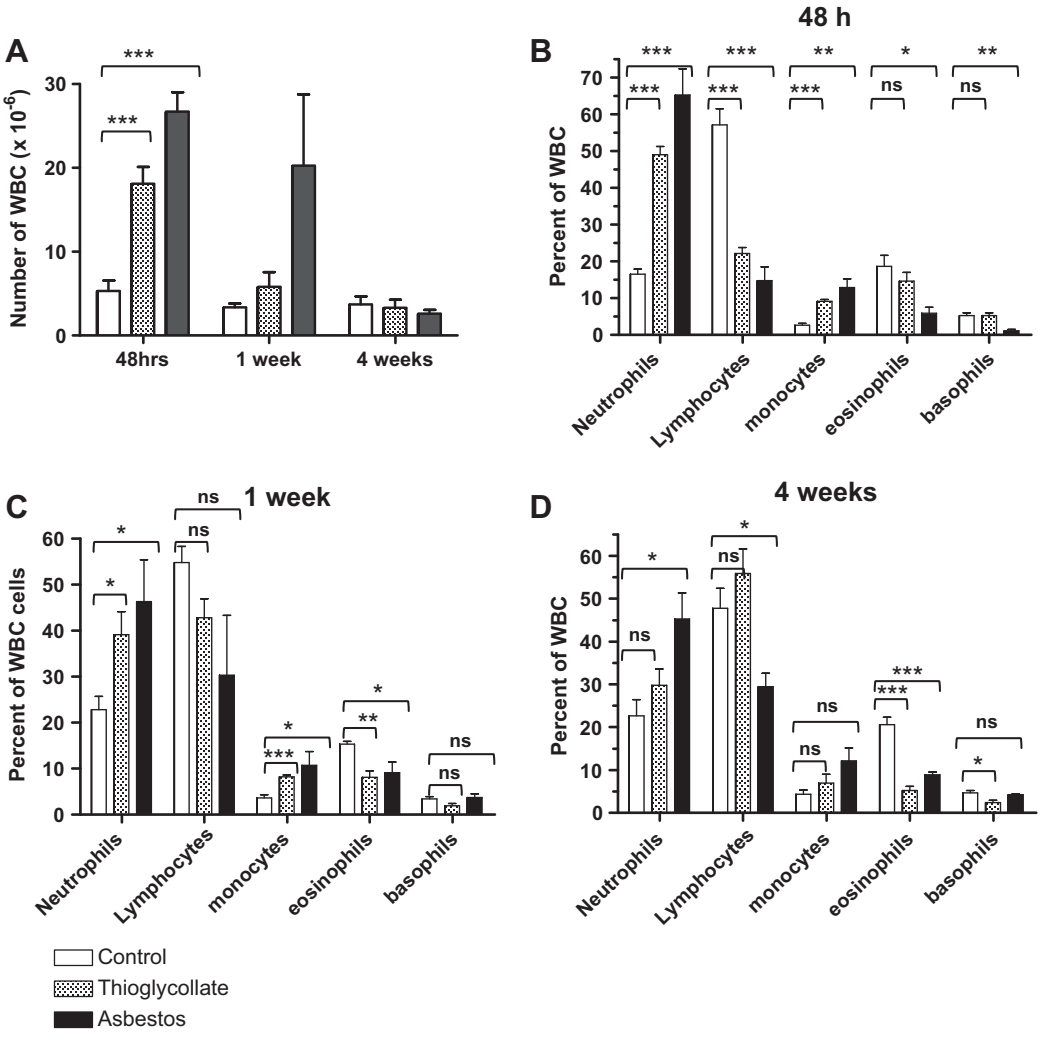

Fig. 2 – Cells infiltrating the peritoneum after i.p. injection of asbestos (black bar), thioglycollate (hatched bar) or PBS control (clear bar); five MexTAg 299h mice were analysed per group. White blood cell counts at 48 h, 1 week and 4 weeks after injections (A). Percentages of neutrophils, lymphocytes, monocytes, eosinophils and basophils 48 h (B), 1 week (C) or 4 weeks (D) after injection. Key for statistical significance: ns: not significant ($p > 0.05$); $^{*}0.05 < p < 0.01$; $^{**}0.01 < p < 0.001$; and $^{***}p < 0.001$.

The count in both thioglycollate- and asbestos-exposed mice increased 3–4-fold from baseline at 48 h post-treatment (Fig. 2A). The number of cells returned to baseline after a week in thioglycollate treated mice, but remained high in asbestos-exposed mice. After 4 weeks, peritoneal white cell counts were normal in all treatment groups.

The distribution of cell types differed in asbestos- and thioglycollate-treated mice compared to control mice (Fig. 2B). At 48 h neutrophils and monocytes numbers were significantly higher in both asbestos and thioglycollate groups compared to controls ($p < 0.0001$ and 0.0006, respectively), while the proportion of lymphocytes was smaller ($p < 0.0001$). There were significantly fewer eosinophils ($p = 0.015$) and basophils ($p = 0.004$) in the asbestos-exposed mice.

After 1 week the distribution pattern of the cell types was similar to that at 48 h, but the differences were less marked and in most cases were no longer significant in thioglycollate-treated mice compared to control mice (Fig. 2C).

After 4 weeks, when the number of infiltrating white cells had returned to normal, the proportions of the major five white blood cell types in thioglycollate-treated mice were within the normal range, with the exception of eosinophils and basophils, which comprised a lower proportion than in PBS-treated mice ($p = 0.02$ and $p < 0.0001$, respectively; Fig. 2D). Asbestos-treated mice still had significantly more neutrophils ($p = 0.02$) and fewer lymphocytes and eosinophils ($p = 0.01$ and 0.0002, respectively) compared to PBS control mice. Thus, whilst both asbestos and thioglycollate induce acute inflammatory infiltrates, asbestos causes chronic changes not seen in thioglycollate-treated animals.

3.3. *MexTAg mice develop mesothelioma after exposure to asbestos but not thioglycollate*

To test if exposure to an inflammatory stimulus other than asbestos induced mesothelioma development in MexTAg mice, thioglycollate broth or asbestos was injected into cohorts of the four MexTAg mouse lines. Mesothelioma incidence after asbestos exposure was TAg dose dependent: from highest to lowest copy number line (299h, 100 copies; 304i, 32 copies; 270i, 15 copies and 266s, single copy) the incidence of mesothelioma was 100%, 93%, 78% and 69%, respectively, compared with 27% in wild-type mice (Fig. 3). At 45 weeks after treatment, none of the thioglycollate- or PBS-treated mice had developed mesothelioma, so were euthanised for autopsy. Table 1 shows the incidence of mesothelioma at 45 weeks after asbestos or thioglycollate compared with control animals. At this time point all of the high copy MexTAg 299h mice exposed to asbestos had developed mesothelioma, whereas none of the thioglycollate-inoculated mice had mesothelioma or any other peritoneal cancer. In the other three MexTAg mouse lines incidence of asbestos-induced mesothelioma at 45 weeks varied according to TAg copy number, as described previously, but again there was no cancer in thioglycollate or control mice. There was no mesothelioma development in the wild-type mice at 45 weeks after asbestos, thioglycollate or PBS inoculation.

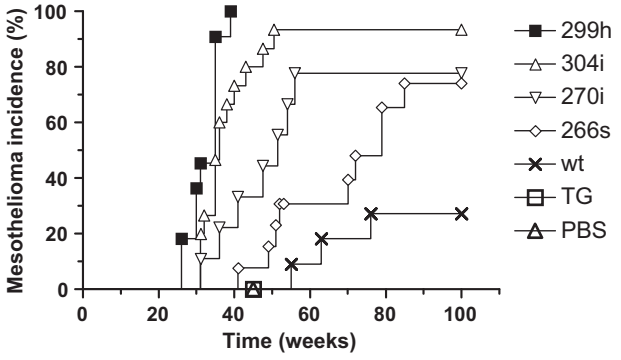

Fig. 3 – Incidence of asbestos-induced mesothelioma in the four MexTAg mouse lines and wild-type mice after i.p. injection with asbestos (10–15 mice per group), thioglycollate (TG) or PBS (5–7 mice per group, for each mouse line). All mouse lines injected with either thioglycollate or PBS were graphed together for simplicity, because mesothelioma did not develop in any mice after either of these agents.

Table 1 – Mesothelioma incidence after asbestos, thioglycollate or PBS peritoneal injection.

Mouse line	Mesothelioma incidence at 45 weeks		
	Asbestos	Thioglycollate	PBS
MexTAg 299h	11/11 (100%)	0/7 (0%)	0/5 (0%)
MexTAg 304i	12/15 (80%)	0/5 (0%)	0/5 (0%)
MexTAg 270i	3/9 (33%)	0/5 (0%)	0/5 (0%)
MexTAg 266s	1/13 (8%)	0/5 (0%)	0/5 (0%)
Wild-type	0/11 (0%)	0/5 (0%)	0/5 (0%)

Note: The table shows the number of mice that had developed mesothelioma 45 weeks after injection with asbestos, thioglycollate or PBS (percentage incidence shown in brackets).

Macroscopically the tissues and organs within the peritoneum of both thioglycollate and control mice had a normal appearance, with no evidence of features previously described in asbestos-exposed mice such as ascites, whitish surface to liver, kidney and pancreas due to reactive mesothelial proliferation; shrunken, nodular liver, adhered intestines and thickening of diaphragm.[7] Furthermore, no macroscopically visible tumours were found within the peritoneum of thioglycollate-treated mice.

3.4. *TAg expression does not affect the rate at which the disease progresses*

TAg binds to and inhibits key tumour suppressor proteins, including the Rb family and p53, and therefore promotes cell proliferation and inhibits apoptosis, two of the hallmark pathways associated with the development of cancer. To determine if the expression of TAg in mesothelial cells altered the rate of progression of disease, we assessed the time between first observation of signs of disease and euthanasia in asbestos-exposed MexTAg and wild-type mice. The time of first observations of disease is also referred to as the diagnosis time. The initial signs of asbestos-induced disease would typically be a swollen abdomen, usually due to ascites accumulation and this includes non-malignant cases. At this stage the mice behave and move normally and there is no

weight loss. Timing of euthanasia was determined by the overall health of the mouse and was dependent on a number of criteria outlined in the approved animal ethics protocol, such as weight loss, ascites accumulation and mouse mobility. We found no relationship between the rate of progression of disease and the level of TAg expression (Fig. 4A). The mean time between first observation of disease and euthanasia for the five groups was not significantly different (one-way analysis of variance $p = 0.1$). Furthermore there was no correlation between TAg copy number and survival after first signs of disease.

The rate of disease progression of the mesothelioma cases (as contrasted with all cases in the above analysis) was compared between groups (Fig. 4B). As expected, there were only three cases of mesothelioma in the wild-type group (approx. 30%). The mean survival time after first observation of signs of disease for these three mice was 134 d. This was a significantly longer survival than the 299h mice (mean time of 73 d, $n = 11$). In an unpaired two-tailed t-test the means between these two groups were significantly different with a p-value of 0.03. However, there was no correlation between TAg copy number (0–100) and median number of days to progression from first diagnosis ($p = 0.94$, $r2 = 0.002$).

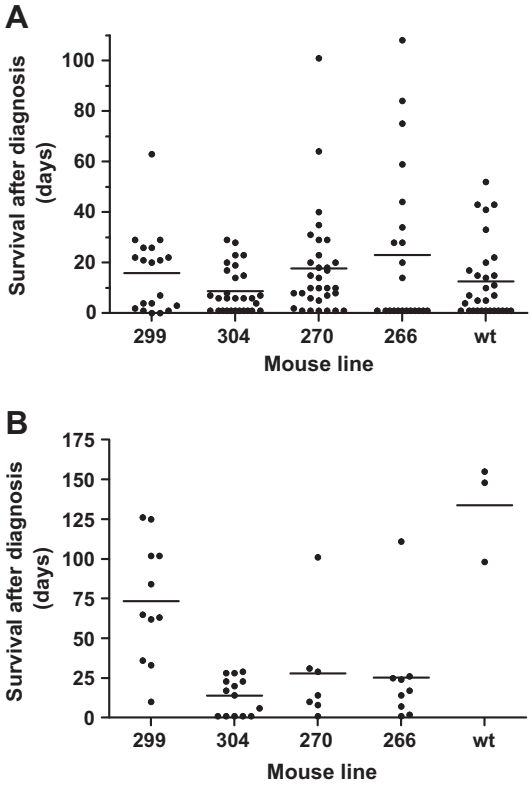

Fig. 4 – Rate of progression of disease after diagnosis is not affected by the amount of TAg expression. (A) Number of days from diagnosis to euthanasia for the four MexTAg mouse lines and wild-type mice, all cases (18–25 mice per group). The MexTAg mouse lines, from highest to lowest TAg copy number (299h, 304i, 270i and 266s), had mean survival of 16, 9, 17 and 23 d, respectively, and for wild-type it was 13 d, one-way Anova test for variance $p = 0.11$. (B) Number of days from diagnosis to euthanasia for mesothelioma cases (12–15 mice per group) in the four MexTAg mouse lines and wild-type mice, mean survival from highest to lowest TAg copy number was 73, 14, 28, 25 and 134 d for wild-type mice, one-way Anova test for variance $p = 0.0003$.

3.5. *Age at first exposure does not affect the rate of disease development nor survival*

People exposed to asbestos when they were over 15 years old have a higher incidence of mesothelioma than those under 15,[10] however, it is possible that these data are biased by asbestos exposure dosage despite allowances made for this in the data analysis. In the MexTAg 299h model the incidence of mesothelioma is 100%, thus we tested whether there was a relationship between age at the time of asbestos exposure and survival time, given a fixed asbestos dose. Asbestos was injected into three groups of MexTAg 299h mice aged 6 weeks, 5–6 months and 8–11 months. Mesothelioma development was independent of mouse age at the time of asbestos exposure (Fig. 5A). Median survival in the three groups in ascending order of age was 33, 35 and 36 months and was not significantly different (log rank test for survival 0.701). Similarly, survival from date of first observation of signs of disease was independent of mouse age at first asbestos exposure (Fig. 5B). Although the figure indicates there was a trend towards longer survival in younger mice it was not significantly different ($p = 0.76$). The mean times between observation of disease and euthanasia for young, middle and old age groups were 25, 17 and 18 weeks, respectively.

In order to further examine the trend to longer survival after diagnosis in younger mice, data from seven experiments using the same dose and schedule of asbestos in MexTAg 299h mice were pooled. The data confirm the above finding that there was no correlation between the age at which mice were exposed to asbestos and survival ($p = 0.14$, Fig. 5C) or the latency period to onset of disease ($p = 0.42$, Fig. 5D).

The pooled data confirmed the trend noted above (Fig. 5B) that there is a significant negative correlation between mouse age at the time of asbestos exposure and time from diagnosis to euthanasia (Pearson's correlate, $r = –0.27$, $p = 0.014$; Fig. 5E), suggesting that older mice do not live as long as younger mice after disease becomes apparent to the observer. Similarly, mice that were older at the time of diagnosis had a poorer prognosis (Fig 5F, $p = 0.0002$, Pearson's correlate $r = –0.41$).

3.6. *Mesothelioma development in MexTAg mice is not affected by gender*

The prognostic significance of gender in the development of mesothelioma after asbestos exposure is controversial.[10–15] The interpretation of epidemiological studies may be confounded by age at the time of exposure, duration of exposure and dose. The MexTAg model allows these factors to be controlled. Age-matched male and female MexTAg 299h mice received the same dose of intraperitoneal asbestos and were monitored for disease development. The rate of development of mesothelioma, the median overall survival (Fig. 6A, $p = 0.67$) and the time from first signs of disease to euthanasia ($p = 0.65$) did not differ between males and females (Fig. 6B).

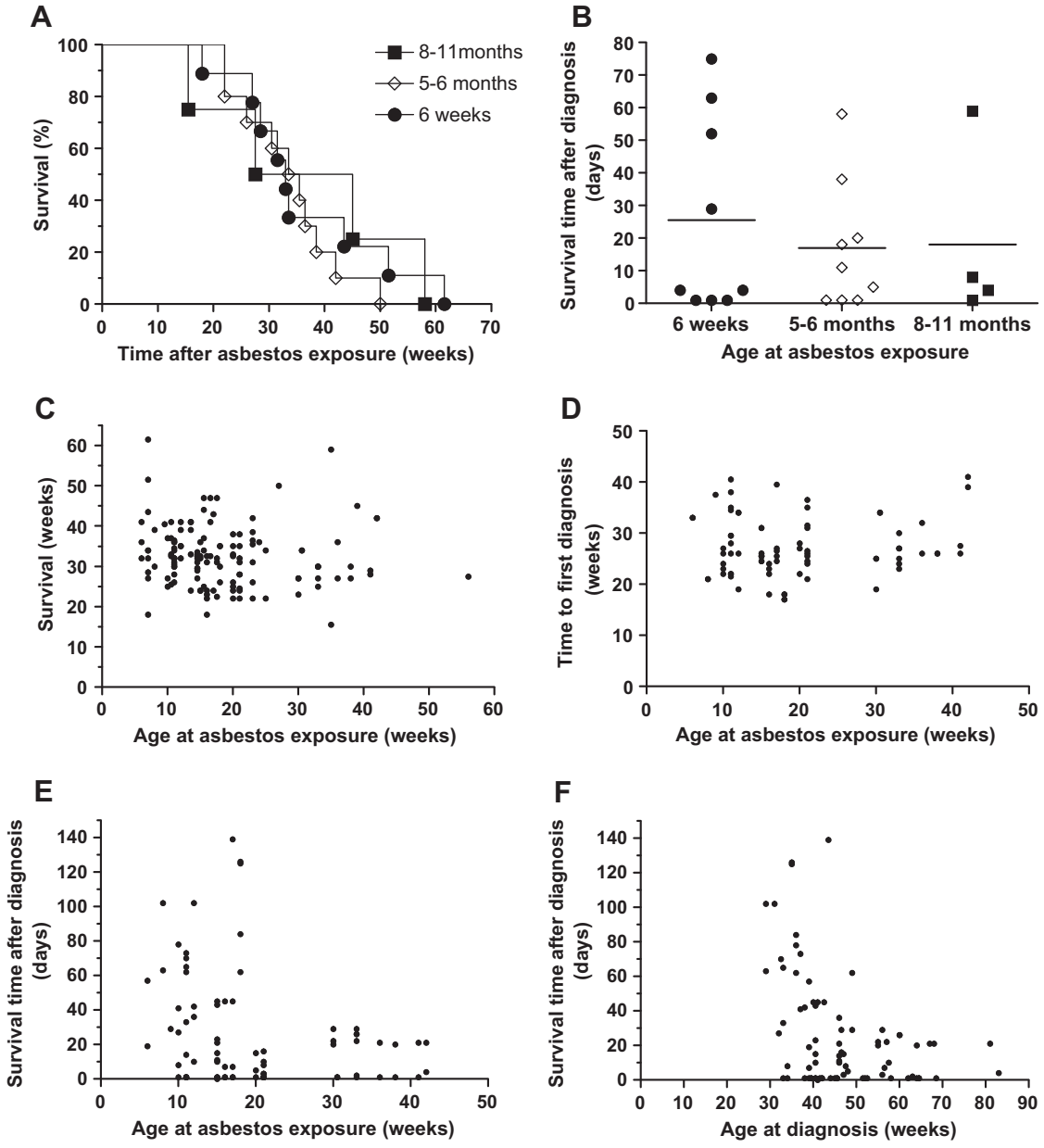

Fig. 5 – Mouse age at the time of asbestos exposure is not a prognostic factor for survival, but does influence the rate of disease progression. (A) Survival after asbestos injection i.p. of MexTAg 299h mice grouped according to age: 6 weeks old (n = 9), 5–6 months old (n = 10) and 8–11 months old (n = 4), log rank test, p = 0.7. (B) Number of days survival after diagnosis for mice in each age group, one-way Anova test for variance, p = 0.76. (C) Data from pooled experiments comparing mouse age on the day of first asbestos injection with the number of weeks survival from the injection date, Pearson's correlate r = –0.094, p = 0.14. (D) Data from pooled experiments comparing mouse age on the day of first asbestos injection with the number of weeks from the injection date to diagnosis, Pearson's correlate r = 0.092, p = 0.42. (E) Mouse age on the day of first asbestos exposure is plotted against number of days survival after diagnosis (data from pooled asbestos experiments). Pearson's correlate r = –0.27, p = 0.014. (F) Mouse age at diagnosis is plotted against number of days survival after diagnosis (data from pooled asbestos experiments). Pearson's correlate r = –0.41, p = 0.0002.

3.7. *Chemotherapy prolongs survival in MexTAg mice*

To demonstrate the utility of MexTAg mice for testing interventions, mice were treated with the cytotoxic drug, gemcitabine. Gemcitabine was selected because firstly, it provides measurable efficacy in human disease and secondly, because we had previously shown that it prolongs survival of animals transplanted with subcutaneous mesotheliomas.[16] Treatment started 16 weeks after the first asbestos exposure, at which time mice appeared healthy but we inferred that they would have incipient disease. Treatment continued until death, typically by euthanasia using the same criteria discussed above. Gemcitabine treatment significantly prolonged survival (p = 0.001) increasing the median survival from 33 weeks in

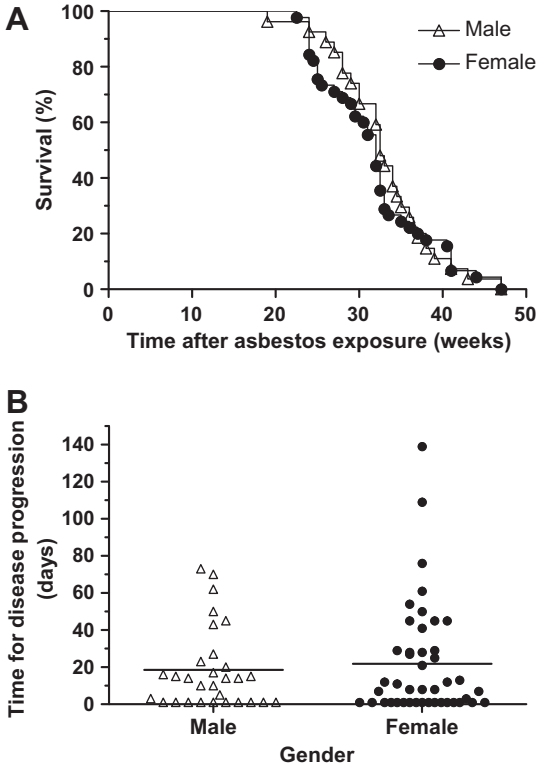

Fig. 6 – Mouse gender does not affect rate of asbestos-induced mesothelioma development. (A) Survival for male ($n = 27$) or female ($n = 45$) MexTAg 299h mice injected i.p. with asbestos, $p = 0.67$. (B) Number of days from diagnosis to end-point for male and female mice, mean progression times are 18.5 and 21.8 d, respectively, $p = 0.65$.

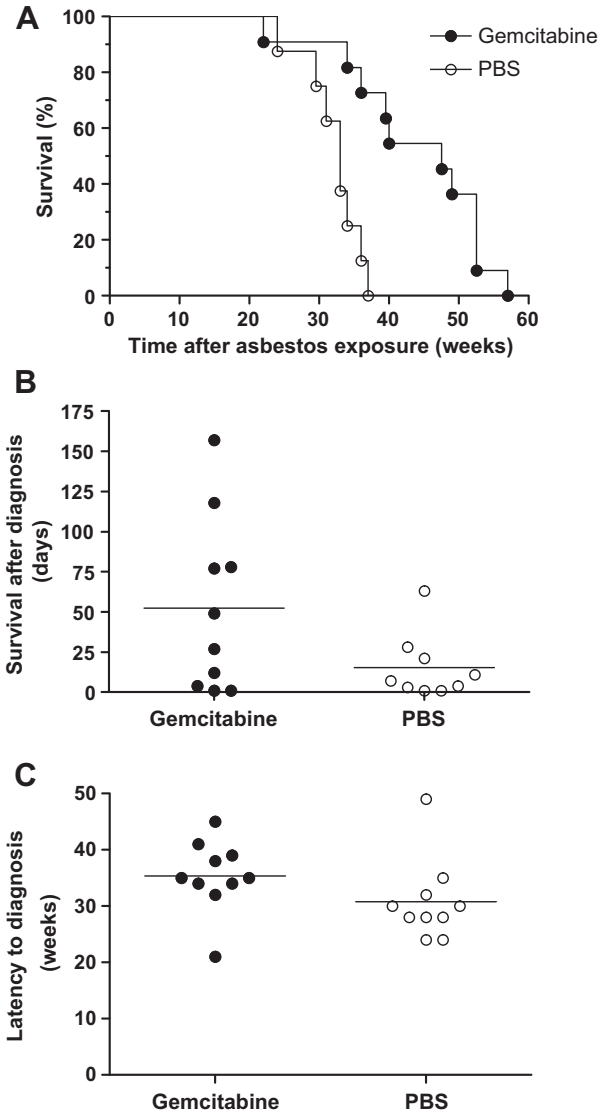

Fig. 7 – Gemcitabine prolongs survival of asbestos-exposed MexTAg mice. (A) Survival curve for MexTAg 299h mice injected i.p. with asbestos and treated with gemcitabine or PBS from week 16 (10 mice per group), log rank test for survival, $p = 0.001$. (B) Mean number of days from diagnosis to end-point for mice treated with gemcitabine or PBS are 52 and 15 d, respectively, $p = 0.052$. (C) Number of weeks from asbestos injection to diagnosis, mean latency period was 35 and 31 weeks for gemcitabine and control groups, respectively, $p = 0.15$.

control mice to 48 weeks in the treatment group (range 24–37 and 22–57 weeks, respectively; Fig. 7A). To explore whether treated animals survived longer before diagnosis or lived longer with established disease, we examined the time between first observation of signs of disease and euthanasia. There was an increase in survival from first signs of disease until euthanasia that approached statistical significance (median 7 and 38 d for control and treatment groups, respectively, $p = 0.052$) (Fig. 7B). However, time from injection of asbestos to first observation of disease ('latency') was not significantly prolonged by gemcitabine treatment, median latency was 35 weeks compared to 29 weeks for the control group (Fig. 7C, $p = 0.15$).

3.8. *COX-2 inhibition does not ameliorate asbestos-induced mesothelioma*

Celecoxib is a non-steroidal anti-inflammatory drug that specifically inhibits COX-2, the key rate-limiting enzyme required for prostaglandin synthesis. COX-2 inhibitors have been reported to have cancer prevention activity and can delay progression of pre-neoplastic lesions in colorectal and prostate cancer.[17,18] COX-2 has a potential role in mesothelioma where it is frequently overexpressed; furthermore, high levels of COX-2 in mesothelioma samples correlate with poor prognosis.[19]

MexTAg 299h mice were fed a diet containing celecoxib from 1 d before the asbestos injection. Serum celecoxib levels were assayed in randomly selected representative mice from the different treatment groups at 10–15 weeks, 20–29 weeks and 30–40 weeks (Fig. 8A). Whilst celecoxib levels were variable, mean levels did not differ over the three time points ($p = 0.5$) and all treated mice had detectable levels of celecoxib. Survival was the same in both groups: median survivals for celecoxib and control groups were 31 and 28 weeks, respectively (Fig. 8B, $p = 0.95$). However, time from first observation of disease to euthanasia was shorter in mice receiving

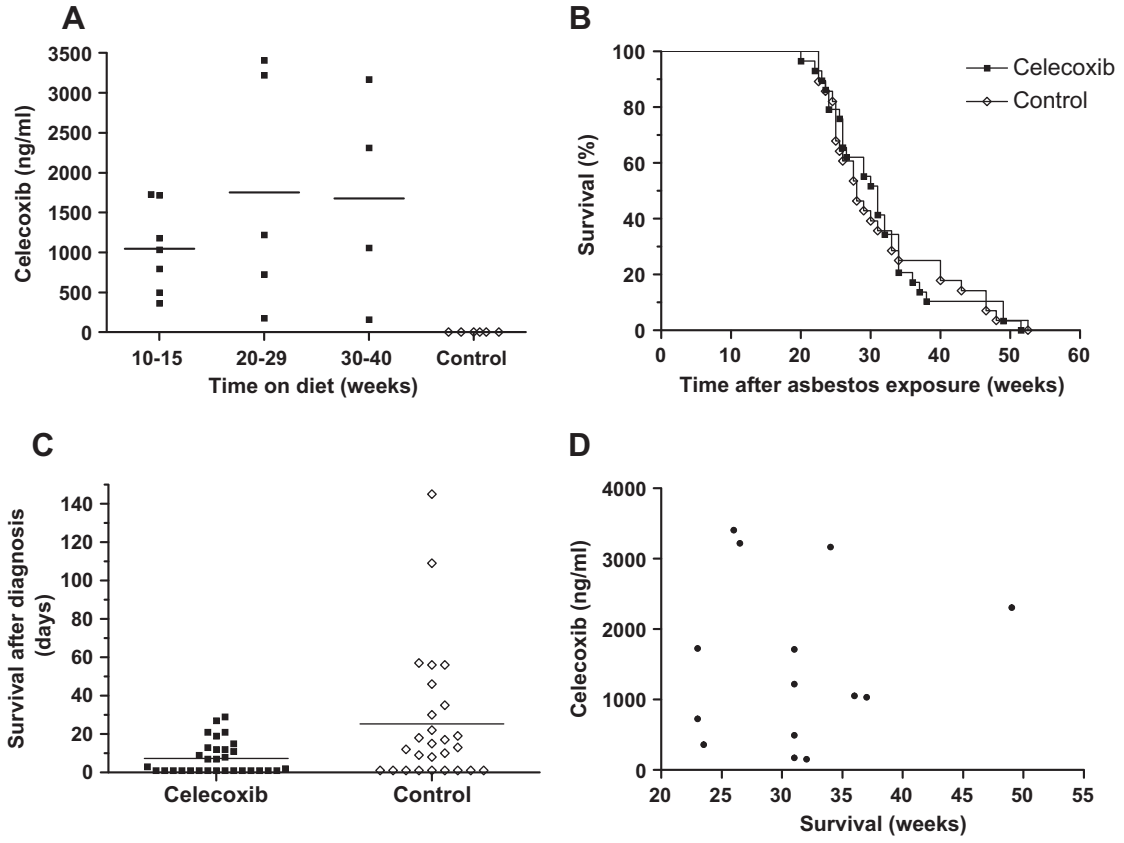

Fig. 8 – Prophylactic treatment with COX-2 inhibitor did not improve survival of asbestos-exposed MexTAg 299h mice (30 mice per group). (A) Serum levels of celecoxib in randomly selected mice, measured at the indicated times after provision of diet. (B) Survival of mice provided with celecoxib or normal diets, log rank test, $p = 0.95$. (C) Number of days survival from diagnosis to end-point, $p = 0.007$. (D) Serum celecoxib level versus survival, $p = 0.77$.

celecoxib than in control mice (2 and 13 d, respectively, $p = 0.007$; Fig. 8C). There was no correlation between serum levels of celecoxib and survival ($p = 0.77$; Fig. 8D).

4. Discussion

In this paper we show how MexTAg mice offer the opportunity to study asbestos-induced mesothelioma developing in the natural location in a uniform way. MexTAg mice are an optimal model system to study many aspects of mesothelioma, replicating key features of the human disease.

Three other heterozygous knockout transgenic systems have been described that model mesothelioma; these target p53, Nf2 and Akt.[20–22] In each case the mice are more susceptible to asbestos-induced mesothelioma than wild-type animals; however, unlike MexTAg mice, not all these mice develop mesothelioma after asbestos exposure. These systems are also limited by spontaneous development of non-mesothelioma tumours. Although approximately 1–2% of high copy MexTAg mice develop subcutaneous sarcomas over 2 years, these tumours do not complicate the asbestos-induced mesothelioma experiments. In the course of our studies we have never seen the development of peritoneal tumours in non-asbestos-exposed mice. Peritoneal tumours that develop in the presence of asbestos have never been characterised as any other tumour type than mesothelioma, despite intensive histological analysis. We have shown that normal mesothelial cells do not express TAg even in the high copy animals unless cell proliferation has been induced, for example, by cell culturing.[7] This most likely explains this lack of spontaneous tumour development.

Large T antigen is used frequently in transgenic mouse cancer models because it is a potent oncogene. There are several reports in the literature that human mesothelioma samples can contain SV40 sequences, reviewed in.[23] However, it is notable that much of the data have been attributed to PCR contamination and antibody cross reactivity and a causative role for SV40 in human mesothelioma is controversial and not supported by epidemiological evidence.[24–26] In at least one study, SV40 positive mesotheliomas have been associated with a poorer prognosis.[27] Thus we assessed whether continued expression of TAg affected mesothelioma development by analysing the time it took for disease to progress from initial signs to the end-point or euthanasia. Standardised criteria were established to define these dates. Survival was variable in both wild-type mice and the four MexTAg lines but was not significantly different between any group. We conclude that disease progression is not affected by expression of TAg in this model.

Interestingly, in the three proven cases of mesothelioma in wild-type animals the time to progression was significantly longer than in MexTAg mice. We do not know whether this

observation will be upheld if repeated with larger groups, but there is a high degree of variability in survival after diagnosis in malignant cases. Overall, the data do not suggest that TAg-positive tumours grow more aggressively than those lacking TAg, rather that TAg is important as a co-carcinogen in the initiation of mesothelioma in this model, and not in the growth rate of the developing tumour.

MexTAg mice could be used to test the contribution of potential co-carcinogens to mesothelioma development; however, in order to be relevant, it was important for us to establish that other peritoneal irritants did not cause mesothelioma. Asbestos causes a chronic inflammatory response which may play a part in mesothelioma development.[9] While there is an initial acute inflammatory response after peritoneal asbestos instillation, this becomes chronic because of the persistence of asbestos fibres, which are too large to be engulfed and cleared by macrophages. However, intraperitoneal thioglycollate, which causes an acute inflammatory response, does not induce cancer suggesting that persistence of the irritant may be an important determinant of disease.

Population studies have suggested that people older than 15 years at the time of exposure to asbestos have a higher incidence of mesothelioma than those who were exposed when they were aged under 15 years.[10] This might be explained if asbestos fibres are cleared from the lung at rates which decline with time after exposure, and if the initial clearance rate is more efficient in younger people.[28] In our experiments asbestos exposure was equal for each mouse age group and was intraperitoneal. Although previous investigators had shown that rodents injected intrapleurally with asbestos at age 10 months developed mesothelioma 4 months earlier than those injected at age 2 months,[29] we found that mouse age at exposure did not alter the rate of mesothelioma development in MexTAg mice. However, disease progression after diagnosis was significantly more rapid in older mice, consistent with findings in mesothelioma patients.[30,31] We think that this might be because older mice or humans are less biologically resilient and therefore more likely to succumb to disease at a lower tumour burden. Consistent with this, mice that were older at the time of diagnosis had a poorer prognosis.

The prognostic significance of gender in the development of mesothelioma after asbestos exposure is similarly controversial.[10–15] Epidemiological data indicate that women are more susceptible to mesothelioma than men.[32,33] However, the interpretation of such studies may be confounded by age at the time of exposure, duration of exposure and in particular dose, which is difficult to determine accurately but is known to influence the incidence of mesothelioma significantly.[34,35] Our experiments, with uniform dosing and age of exposure, did not show a difference in the rate of development of mesothelioma between genders in MexTAg mice.

We have demonstrated the use of MexTAg mice to test preventative and therapeutic intervention. Many people know that they have been exposed to asbestos, thus the identification of a preventative agent would have a substantial impact. Nevertheless, testing of candidate agents is limited by the relative infrequency of the disease and the large numbers required for such clinical trials. We found no survival benefit from prophylactic COX-2 inhibition in the asbestos-exposed MexTAg mice. It is notable that survival after diagnosis was significantly shorter in the celecoxib group. Although autopsy did not reveal any adverse effects of celecoxib and the usual asbestos-induced changes were apparent, it is possible that inhibition of COX-2 promotes tumour growth once it has reached the malignant stage, possibly involving COX-2 effects on p53-mediated apoptosis and p53 regulation of COX-2 expression[36] as well as a potential synergistic effect with TAg, which is well known to directly inhibit p53.[37] While previous research suggested that COX-2 inhibitors were a chemoprevention candidate for mesothelioma, including an association between COX-2 expression and poor prognosis,[19,27] the notion that COX-2 may confer a survival advantage is supported in a more recent study of 86 mesothelioma samples.[38]

A further test of the fidelity of the model was its ability to approximate the response to treatments seen in patients.[39] We tested the cytotoxic agent gemcitabine, a nucleoside analogue, which has documented activity in human mesothelioma.[40–42] The survival of gemcitabine-treated mice was significantly improved by a median of 15 weeks. Interestingly, survival after diagnosis was significantly longer in the gemcitabine group, whereas survival to onset of disease was not. This suggests a beneficial role for this cytotoxic drug in slowing disease progression. Whilst gemcitabine has not been tested in the phase III setting, cisplatin and pemetrexed, have shown improvements in survival with a similar magnitude in phase III trials.[43,44] We think that MexTAg mice could also be used to test the interactions of combinations of therapies and to investigate the optimal dosing and scheduling of such combinations.[45] In summary, the MexTAg model is robust and suitable for a range of translational experimental work with direct relevance to human mesothelioma.

Conflict of interest statement

None declared.

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