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STRESS INFLUENCE ON DEVELOPMENT OF HEPATOCELLULAR TUMORS IN TRANSGENIC MICE OVEREXPRESSING TGFα

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We investigated whether stress increases tumorigenesis in male transgenic mice that overexpress the gene encoding human transforming growth factor α (TGFα). At the age of 10–15 months, these mice begin to develop spontaneous hepatocellular carcinomas at high incidence. The male TGFα mice were housed with their siblings (non-stressful environment), housed in social isolation, or housed with aggressive non-siblings (stressful environment). Some animals in each group were exposed once a week to a second stressor (swim stress), beginning at the age of 7 months. Housing with aggressive non-siblings increased neoplastic growth in the male TGFα mice: the incidence and multiplicity of liver tumors, and tumor burden were higher in these animals than in the sibling-housed mice. Among the isolated TGFα mice, only the tumor burden was increased, when compared with the sibling-housed TGFα mice. Swim stress significantly increased the incidence of liver tumors and tumor burden in the sibling-housed TGFα mice. Plasma levels of 17β-estradiol (E2) that are elevated in the TGFα mice, were modestly but significantly higher in the non-sibling housed transgenic mice than in the sibling-housed. Natural killer (NK) cell activity, reduced in these mice, was not affected by housing environment. These data suggest that stress promotes the growth of hepatocellular tumors in the male TGFα mice. Whether estrogens are involved in mediating this association remains to be determined.

An increasing amount of human and animal data suggest the importance of psychosocial factors in vulnerability to cancer, and length of survival once a particular tumor has developed (1–3). An ability to cope with stress appears critical: those individuals that are not able to cope with stress show an increased risk to develop cancer (4–6). However, some human studies contradict the importance of stress on cancer (for review, see (1)). Individuals that are exposed to a limited number of life-stressors (e.g., marriage, divorce, illness, death) are at higher risk to develop cancer than individuals that are exposed to several life-stressors (7–10).

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We hypothesized that the male CD-1 MT42 transgenic mice, which overexpress transforming growth factor α (TGFα) in multiple tissues throughout their life (11), might be particularly susceptible to psychosocial stressors. The most pronounced effect of expression of this transgene is the development of a high incidence of spontaneous hepatocellular carcinomas in 10–15-month-old male TGFα mice (11). Female MT42 TGFα mice do not exhibit an increased incidence of malignancies (11). Several months prior to the appearance of liver tumors, these mice exhibit lengthened immobility in the swim test, indicating an impaired ability to cope with stress, and increased aggressive behavior (12). They also show a four-fold increase in plasma 17β-estradiol (E2) levels, and a 25% reduction in natural killer (NK) cell activity (12). Both estrogen and NK cell activity are associated with hepatocellular carcinoma (13–16), and they are influenced by stress (17–22).

The present study examined whether psychosocial manipulations affect liver tumorigenesis in the male TGFα mice. Individual housing has been reported to increase

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experimentally-induced tumor growth. Dechambre & Gosse (23) and Sklar & Anisman (24) observed an enhanced growth of several forms of rapidly growing inoculated tumors in singly housed male and female mice. Weinberg & Emerman (25) reported that male mice that were reared in a sibling group, but singly housed following mammary tumor cell injection (SC115), showed significantly increased tumorigenesis compared with mice housed with their siblings. Those mice that were first reared individually and then moved to a larger group exhibited markedly reduced mammary tumor growth. This was particularly true for mice that started to fight following group-housing (24, 25).

We chose individual housing and group-housing with male mice which were not littermates (non-siblings), as the psychosocial manipulations in our attempts to influence liver tumor onset and growth in the TGFα mice. Housing with non-siblings is stressful to male TGFα mice because the male TGFα mice are overly aggressive, and fight with non-sibling cage-mates (12). The fighting occurs randomly, and the mice have no means to control or avoid it. To examine whether other stressors might intervene with the effects of social stress on tumorigenesis, some transgenic TGFα mice housed singly, or with siblings or non-siblings, were exposed to an additional stressor (swim stress). The possible mechanism of action of stress on tumorigenesis in the male TGFα mice was investigated by determining the effects of different housing environments on serum E2 levels and splenic NK activity.

Material and Methods

Mice of the CD-1 background were made transgenic for the growth factor TGFα (11) and provided by Dr. Glenn Merlino from the National Cancer Institute in Bethesda, USA. The generation and characterization of these animals has been previously described (11). The animals used in the present study were bred in Georgetown University. They were maintained on a 12 h light–12 h dark cycle and allowed ad libitum access to food and water.

The TGFα mice were housed with their siblings until they were 4 months old. The TGFα mice were then assigned into three groups: a) individually housed mice, b) mice housed with siblings, and c) mice housed with unfamiliar cage-mates (n = approximately 40 mice per group). Some of these mice died before they reached the age of 11-months when all the remaining mice were sacrificed for tumor assays. Among the group-housed mice, 3–4 animals were kept in each cage to avoid serious fighting among the non-sibling housed males. At the age of 11 months, the mice were sacrificed for the collection of blood for hormonal measurements, spleens for measurements of NK cell activity, and livers for the determination of the appearance of hepatocarcionomas.

Swim stress. At the age of 7 months, some individually housed, sibling housed and non-sibling housed mice were either exposed to swim stress weekly or left undisturbed (n = 10–15 per group). Some of these animals were accidentally sacrificed during the course of the study, leaving only 5–11 mice per group. An animal was placed individually in a plastic cylinder (height 17 cm, diameter 21 cm) containing 8 cm of water maintained at about 25°C for 5–10 min. Swim stress continued until the animals were sacrificed for hormone, immune and liver growth measurements.

Measurement of plasma E2 levels. When the animals were 11 months old, 5–9 TGFα mice from each group were anesthetized using methoxyflurane inhalant to collect their blood by using cardiac puncture. The blood was placed in tubes, centrifuged, and stored at −20°C. The plasma E2 levels were then determined by using a specific double antibody kit No. 07-138102 from ICN Biomedicals, Inc. (Irvine, CA) according to the manufacturer's instructions.

Measurement of NK activity. Dr. Prince Arora from NIH used five mice from each group to determine splenic NK cell activity. The male TGFα mice were sacrificed by cervical dislocation, their spleens were removed, and placed in Hanks' balanced salt solution containing 10% heat activated fetal bovine serum. Single cell suspensions were prepared as described by Arora & Shearer (26). NK cell activities were assayed as described by Arora & Shearer (27) and Fride et al. (28). YAC-1 tumor cells were used as targets for NK-mediated cytolysis. Target cells were labeled with 200 μCi: $Na_2{}^{51}CrO_4$ (Dupont-New England Nuclear, Boston, MA), washed twice in HBSS containing 10% HFBS and 3 ml Hepes buffer (GIBCO). After counting, target cells were added (100 μl) to microtiter wells containing effector spleen cells, such that different effector:target cell ratios were evaluated. The plates were centrifuged for 3 min at 400 rpm and incubated at 37°C for 4 h in a 95% air–5% CO_2 atmosphere. After incubation, the plates were centrifuged for 3 min at 800 rpm and the supernatant was collected with a Titertek Supernatant Collection System (Skatron, Inc., Sterling, VA) and counted in a Beckman Auto Gammascintillation spectrometer. The percentage of lysis was determined as described by Arora & Shearer (27).

Measurement of tumor development. After the plasma and spleens were taken, the animals were decapitated by cervical dislocation and their livers were removed. The livers were weighed and placed in formalin. Gross morphological and histological examination was performed by a pathologist (Dr. Soon Paik, Georgetown University). He counted the number of single or multifocal liver nodules and measured their size with a caliber. No staining was done since it has been previously described in detail that all liver nodules in the transgenic TGFα mice are well-differentiated hepatocellular carcinomas of variable size displaying either solid or trabecular pattern (11, 29).

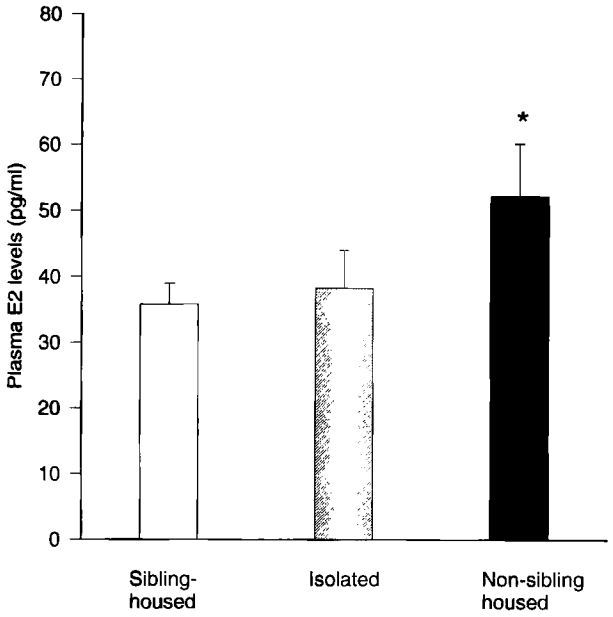

Fig. 1. Plasma E2 levels in the 11-month-old TGFα mice housed in social isolation or with their siblings or non-sibling TGFα mice. The means ± SEM of 5–9 animals per group are shown. Significantly different from sibling-housed mice: *$p < 0.05$.

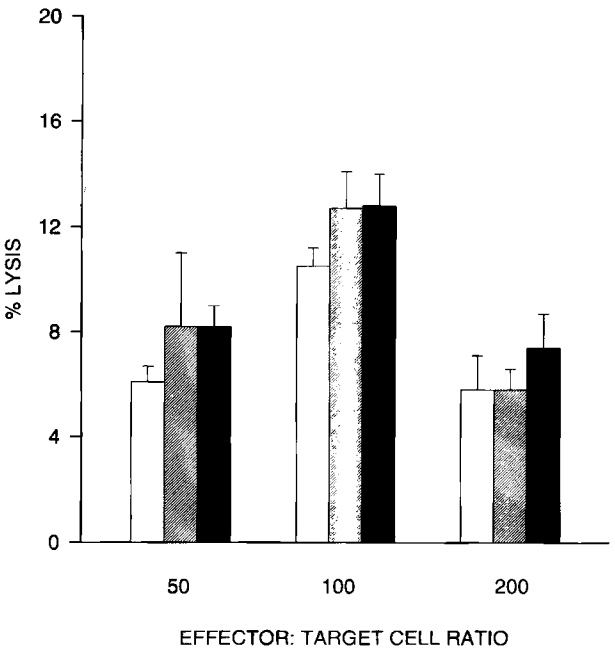

Fig. 2. NK cell activity using three different effector:target cell ratios in the 11-month-old TGFα mice housed in social isolation or with siblings or non-siblings. Spleen cells were the effects and YAC-1 cells were used as targets. The means ± SEM of 5 mice per group are shown. □: sibling-housed; ▨: isolated; ■: non-sibling housed.

Statistical analysis. Statistical tests were done using the SOLO statistical program (BMDP Statistical Software, Los Angeles, CA, USA). Results for the body weight, plasma E2 levels and number of tumor nodules per liver, and liver weights were analyzed using one-way ANOVA. Data for NK cell activity were analysed using two-way ANOVA. Between-group comparisons were made with Fisher's least significant difference test. Because the data for the tumor incidence per group and tumor volume did not conform to a normal distribution, they were analysed using non-parametric χ^2 test and Kruskal-Wallis test respectively. Between-group comparisons were made with Dunn's test.

Results

Plasma E2 levels. Among the transgenic TGFα mice, different housing environments failed to have a significant effect on serum E2 levels ($F(2,15) = 2.35$, $p < 0.105$) (Fig. 1). However, a post-hoc analysis done using Fisher's least significant difference test revealed that the non-sibling housed mice had significantly higher plasma E2 levels than the sibling-housed male TGFα mice ($p < 0.05$).

NK cell activity. NK cell activity in the spleen was not significantly altered due to different housing environments in the male TGFα mice (Fig. 2).

Liver tumors

Housing stress. Different housing environments had a significant effect on the hepatocellular tumor incidence in the transgenic TGFα mice ($\chi^2 = 10.09$, $df = 2$, $p < 0.01$). At the age of 11 months, 7 of the 19 TGFα mice housed with their siblings, 10 of the 19 isolated TGFα mice, and 15 of the 17 TGFα mice housed with non-siblings had hepatocellular carcinomas (Table 1). The multiplicity of carcinomas (number of tumor nodules per liver) was higher in the non-sibling housed TGFα ($p < 0.05$) than in the sibling-housed mice ($F(2,53) = 3.39$, $p < 0.04$) (Table 1).

The liver weight was used as an index of tumor burden. Measurement of liver weights indicated that the TGFα mice housed with non-siblings had significantly heavier livers than the sibling-housed TGFα mice ($p < 0.05$) ($F(2, 50) = 2.24$, $p < 0.02$) (Table 1). Measurement of the mean volume of liver carcinomas revealed that the tumors were smallest in the sibling-housed transgenics, but the difference failed to reach statistical significance ($H = 4.20$, $df = 2$, $p < 0.12$) (Table 1).

Swim stress. Swim stress had a significant effect upon tumorigenesis in the sibling-housed male TGFα, ($\chi^2 = 15.7$, $df = 5$, $p < 0.01$) (Table 2). Among the transgenic animals not exposed to swim stress, only 1 of 11 sibling-housed mice had developed tumors at the age of 11 months. In contrast, the tumor incidence was 5 of 7 sibling-housed animals among the swim-stressed group. All 5 mice housed with non-siblings and 4 of 7 isolated mice that were not exposed to swim stress had developed liver tumors. The corresponding numbers among the

Table 1

Incidence of hepatocellular carcinomas in the 11-month-old TGFα mice exposed to different housing environments

	Sibling-housed (n = 19)	Transgenic TGFα mice	
		Isolated (n = 19)	Non-sibling housed (n = 17)
Number of animals with tumors	7	10	15
Percentage of animals with liver tumors	37	53	88
Total number of tumors	12	14	21
Number of liver nodules/mouse[1]	0.6 ± 0.2	0.7 ± 0.2	1.3 ± 0.2*
Volume of liver nodules (cm^3)	0.6 ± 0.4	2.3 ± 1.2	2.7 ± 1.0
Liver weight (g)	5.6 ± 0.7	7.8 ± 1.0*	9.9 ± 1.4*

[1] Mean ± SEM per group.
Statistically significant differences, when compared with sibling-housed TGFα mice: *$p < 0.05$.

swim-stressed mice were 5 of 7 in the non-sibling housed and 2 of 6 in the isolated TGFα mice.

There was a significant interaction between swim stress and tumor burden among the male TGFα mice ($F(2, 34) = 5.59$, $p < 0.008$) (Table 2). The sibling-housed TGFα mice showed significantly higher tumor burden following swim stress than when not stressed ($p < 0.01$). Swim-stress did not significantly alter tumor burden in the non-sibling housed or isolated transgenic mice (Table 2).

Discussion

In the present study, housing with aggressive cage-mates affected liver tumorigenesis in the male TGFα mice. The non-sibling housed transgenic mice that were forced to share a home cage with non-familiar and aggressive male mice, exhibited higher liver tumor incidence and multiplicity, and tumor burden than the sibling-housed TGFα mice. The findings do not appear to be caused by physical effects of fighting. Immediately following new housing arrangements, non-sibling housed mice started to fight. The fighting became less frequent by time, and when the non-sibling housed mice were sacrificed to determine the status of liver tumorigenesis, none or only minor fight-marks were seen. Thus, the difference in tumor growth between the non-sibling and sibling-housed mice may be related to the stress associated with being housed with non-familiar aggressive mice.

A number of earlier studies have investigated the effect of isolated housing on the growth of transplantable tumors or inoculated tumor cells in mice. For example, male and female mice have been isolated immediately following an inoculation of melanoma B16, leukemia L1210, Krebs-2 ascites, P815 mastocytoma cells or SC115 mouse mammary tumors (23–25). After approximately 10 days tumors become noticeable. The tumors appeared earlier and grew faster in isolated mice, than in either non-sibling (23) or sibling-housed mice (25). Our data with spontaneously arising tumors in immunocompetent mice partly support, and partly are at variance with, these earlier findings (23–25). In the male TGFα mice, social isolation lasting for 7 months did not influence tumor incidence or multiplicity when compared with sibling-housed mice. Tumor burden was higher in the isolated mice. The cause for the opposing results in the previous and present studies might be related to the length of the isolation period. In the earlier studies, the isolation period was relatively short (less than 4 weeks) (23–25). In contrast, in our study housing manipulations lasted for 7 months. Acute isolation may have opposite effects than chronic social isolation on hormonal, immune and behavioral parameters (30). Another critical difference between the present and previous studies is that the earlier studies used rapidly proliferating xenografts or allografts (23–25), whereas the tumors

Table 2

Incidence of hepatocellular carcinomas in the 11-month-old TGFα mice exposed to swim stress and different housing environments

	Sibling-housed		Isolated		Non-sibling housed	
	Stressed (n = 7)	Controls (n = 11)	Stressed (n = 6)	Controls (n = 7)	Stressed (n = 7)	Controls (n = 5)
Number of animals with tumors	5	1	2	4	5	5
Percentage of animals with liver tumors	71	9	33	57	71	100
Liver weight (g)[1]	5.5 ± 1.7*	2.5 ± 0.1	3.0 ± 0.2	3.7 ± 0.3	3.5 ± 0.5	4.8 ± 0.9

[1] Mean ± SEM per group.
Statistically significant differences, when compared with sibling-housed TGFα mice: *$p < 0.05$.

in the male TGFα mice were slowly growing spontaneous hepatocellular tumors. Thus, our results indicate that social isolation does not shorten latency of tumors or increase tumor incidence in the male transgenic TGFα mice. As an explanation of the differences in tumor burden, it is evident that once the tumors appear they may grow faster in the isolated TGFα mice than in the sibling-housed mice.

Housing environment, as well as swim stress affected tumorigenesis. The sibling-housed transgenic mice that were exposed to swim stress had a higher incidence of hepatocellular tumors and increased tumor burden when compared with sibling-housed TGFα mice that were not exposed to swim stress. Tumor incidence or burden was not significantly influenced by swim stress in the non-sibling housed or isolated transgenic mice. Due to the low number of mice included into the swim stress study, however, these results remain preliminary.

The mechanism mediating the association between stress and liver tumorigenesis in the male TGFα mice does not appear to be related to NK cell activity. NK cell activity was not affected by housing environments among the male transgenic TGFα mice. Similar negative results were obtained in our unpublished study (Hilakivi-Clarke & Arora) in which we compared NK cell activity in non-transgenic male CD-1 mice housed in social isolation or in groups of 3–4 littermates for 8 weeks. However, it is possible that NK cell activity was altered shortly after the mice were assigned into the different housing environments, and the activity returned to normal levels during the 7-month period of housing with siblings, non-siblings or in social isolation.

In many studies, estrogen has been shown to regulate hepatocellular carcinoma (14, 15). In particular, castration of male transgenic TGFα mice decreases the incidence of tumor nodules by 7-fold (16). In the present study, housing with non-siblings caused a modest but significant elevation in the plasma E2 levels. However, the plasma E2 levels did not differ significantly between the TGFα mice which had developed hepatocellular carcinomas at the age of 11-months (43.2 ± 6.3 pg/ml) and those which had not (37.9 ± 3.2 pg/ml). Further, the plasma E2 levels did not correlate significantly with tumor burden (Pearson test; $r = 0.34$, $df = 18$, NS) or tumor incidence ($r = 0.09$, $df = 18$, NS). Thus, the mechanism of action of stress on hepatocellular tumors is not clear.

Taken together, stress increases the development of hepatocellular carcinomas in the male TGFα mice. Since the findings in humans also suggest that stressors and poor ability to cope with stress may increase the risk of developing tumors and shorten survival (4–6), stress may activate the inherited or acquired molecular events that initiate/promote neoplastic transformation. The nature of the biological mechanisms remains to be determined.

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