Title

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Journal

Anesthesia & Analgesia, 126(6)

ISSN

0003-2999

Authors

Meier, Angela
Gross, Emilie TE
Schilling, Jan M
et al.

Publication Date

2018-06-01

DOI

10.1213/ane.0000000000002902

Peer reviewed

HHS Public Access

Author manuscript

Anesth Analg. Author manuscript; available in PMC 2019 June 01.

Published in final edited form as:

Anesth Analg. 2018 June ; 126(6): 1910–1913. doi:10.1213/ANE.0000000000002902.

Isoflurane Impacts Murine Melanoma Growth in a Sex Specific, Immune-Dependent Manner: A Brief Report

Angela Meier, MD PhD[1], **Emilie T. E. Gross, PhD**[2], **Jan M. Schilling, MD**[1,4], **Ruth Seelige, PhD**[2], **Yujin Jung**[2], **Endi Santosa, MS**[2], **Stephen Searles**[2], **Tuo Lin**[3], **Xin M. Tu**[3], **Hemal H. Patel, PhD**[1,4], and **Jack D. Bui, MD PhD**[2]

[1]Department of Anesthesiology, University of California San Diego, San Diego, CA, USA

[2]Department of Pathology, University of California San Diego, San Diego, CA, USA

[3]Departmetn of Family Medicine and Public Health, University of California San Diego, San Diego, CA, USA

[4]Department of Veterinary Affairs, San Diego Health Care System, San Diego, CA, USA

Abstract

The impact of volatile anesthetics on cancer progression has been observed for decades, but sex differences have not been described. Male and female immune systems vary considerably, and the immune system plays an important role in limiting cancer growth. Currently, mouse models describing the impact of volatile anesthetics on cancer growth are limited to same-sex models. In this brief report, we describe a sex specific impact of isoflurane on melanoma growth observed in wild-type but not in immune-deficient mice. Future experimental designs related to anesthesia and cancer should evaluate the biological variable of sex in a systematic manner.

Introduction

Emerging clinical data highlight the importance of anesthetic choice during tumor surgery on subsequent cancer survival[1]. Specifically, the use of inhalational anesthesia has been suggested to hasten death in cancer patients[1]. The contribution of the immune system in controlling tumor growth is considered a “hallmark of cancer”[2] and inhalational anesthetics

Angela Meier: This author designed the experiments, conducted experiments, collected, assembled and analyzed the data, wrote the manuscript
Emilie T. E. Gross: This author helped with murine experiments, edited manuscript
Jan M. Schilling: This author helped with experimental setup (isoflurane chamber), statistical analysis and edited manuscript
Ruth Seelige: this author helped with experiments, edited manuscript
Yujin Jung: this author helped with experiments
Endi Santosa: this author helped with experiments
Stephen Searles: this author helped with experiments
Tuo Lin: Performed statistical analysis of all data
Xin M. Tu: Performed statistical analysis of all data, provided critical feedback and edited the manuscript
Hemal H. Patel: edited the manuscript and provided critical feedback
Jack D. Bui: this author helped design and interpret the experiments, provided mentorship, provided lab space and edited manuscript, provided critical feedback

Conflicts of Interest
None

can significantly modulate the immune response[3]. Biological differences between men and women are plentiful and complex, and immune related sex differences directly translate into differences in human disease incidence and survival[4]. However, studies describing the interactions of anesthesia and the immune system have not taken sex into consideration, and rodent models in this field of study use same sex animals only[5] or do not specify sex[6]. Here we communicate our observation that isoflurane has a sex-specific and immune-dependent effect on murine melanoma growth, impacting male but not female tumor growth. Sex differences should be taken into consideration when studying the impact of inhalational anesthesia on cancer progression.

Methods

The UCSD IACUC approved all of the described animal studies. Animals: Animals used in our studies were either bred at our facility or ordered from Charles River. They were provided with food and water ad libitum. Experiments: *In vivo* anesthetic exposure: For tumor growth experiments, male and female wild-type (WT) C57BL/6 mice, male C57BL/6 RAG1$^{-/-}$ or male C57BL/6 RAG2$^{-/-}$ x $\gamma_c^{-/-}$ mice between the ages of 8–12 weeks were injected subcutaneously into the right flank with 1×10^6 cells of the melanoma cell line B16F1ova harvested at approximately 80% confluence. After injection, mice were anesthetized with 1–1.5% of vaporized isoflurane (Fluriso, Vet One, M1000 or SurgiVet Vaporizor) in oxygen in a Plexiglas chamber while continuously monitored. Their body temperature was maintained by using a temperature-controlled pad. Mice were subsequently emerged and kept at our facility for the remainder of the experimental time as described above. Tumors were measured blinded whenever possible on day 7, 10 and 13/14 unless the tumor size exceeded the permitted parameters by our animal protection protocol in which case the animals were sacrificed before the end of the experiment to alleviate suffering. Tumor size was recoded on the day of assessment and/or day of sacrifice. *In vitro* anesthetic exposure: For examining the impact of isoflurane on the tumor directly B16 melanoma cells were exposed either for 2hrs to 1–1.5% of isoflurane in an incubator chamber with continuous 5%CO_2 air mix gas flow (Billipus-Rothenberg) or to gas flow without isoflurane, and subsequently injected into male and female C57BL/6 wild-type mice.

Statistics

Changes of tumor size over time and difference of such changes between experimental and control as well as between male and female mice were modeled using the generalized estimating equations (GEE), in which treatment (Isoflurane vs. control, with control serving as the reference group), time (day 7, 10 and 14, with day 7 serving as reference level), gender (male vs. female, with female serving as reference level) and their interactions formed the predictors. If there were significant interactions between two (or three) factors, we assessed factor effects within levels of the interacting factor(s). If no significant interaction was present between any factors, we reported main effects for each factor collapsing over the other factors. The semi-parametric GEE requires no distribution assumption, providing valid inference for a broad class of data distribution[11]. All analyses were set at type I error alpha = 0.05.

Prior to fitting the GEE, missing data were imputed for those that were sacrificed due to large tumors using the largest observed tumor size at assessments on day of their sacrifice (n = 1 in the control group and n= 4 in the isoflurane group for the WT *in vivo* experiments and n = 1 in the female control group in the *in vitro* experiments). Missing data due to any other reason unrelated to tumor growth were not imputed (n = 1 for one mouse in the male *in vivo* control group who was sacrificed due to fighting injuries). We also imputed data for the mice that were sacrificed using the last observation carried forward method. Since results from the two methods are quite similar, we only report the ones from the first approach.

The sample size of the male mice was able to detect a large between-group effect size (Cohen's d = 0.88) with 80% power and a two-sided alpha = 0.05. The actual effect size observed in our data was d = 0.96 for the difference between the isoflurane exposed and control group at day 14 within the male mice, slightly exceeding the detectable effect size. Power was actually larger than indicated by the power analysis because of modeling the repeated assessments using the GEE.

Results

Overall, we observed that melanoma grew faster in male mice treated with isoflurane compared to control male mice. This effect was not seen in female mice. Given the potential impact of time, gender, and treatment group, we proceeded to perform a GEE analysis to formally test the impact of these parameters on tumor growth rate. The GEE showed significant main effects ($p < 0.001$ for time, $p = 0.003$ for treatment groups, and $p < 0.001$ for gender), two-way interactions ($p < 0.05$ for time by treatment, $p < 0.001$ for time by gender and $p = 0.016$ for treatment by gender), and three way interaction ($p = 0.038$). As expected, tumor size increased significantly over time (significant increase from day 7 to day 10 ($p < 0.001$, CI =(38.6, 78.0)) and to day 14 ($p < 0.001$, CI = (42.7, 121.5)) but no significant difference in tumor growth was seen between the male and female mice in the control group [$p = 0.37$, CI = (−40.4, 25.1) at day 10, and $p = 0.89$, CI = (−15.3, 108.5) at day 14]. Within the females, there was no significant difference between the isoflurane exposed and control group ($p = 0.37$, CI = (−34.4, 12.8) at day 10, and $p = 0.89$, CI = (−46.3, 53.1) at day 14). Within the male group, there was a significantly higher increase in the isoflurane group [$p = 0.01$, CI = (13.7, 107.7) at day 10, and $p = 0.042$, CI = (2.9, 159.1) at day 14, compared to the control. The specific effect of isoflurane in male but not in female mice is indicated in Figure 1A, which shows observed tumor size (without any imputed data) for the control vs. isoflurane exposed mice. This effect of isoflurane on melanoma growth was absent in male mice lacking functional B and T cells ($RAG1^{-/-}$ in Figure 1B) or in mail mice lacking functional B, T and NK cells ($RAG2^{-/-}$ x $\gamma_c^{-/-}$, Figure 1C), suggesting that the intact male immune system participated in translating the isoflurane exposure to a clinical phenotype.

To further corroborate this concept, the tumor cells were exposed to 2hrs of 1–1.5% isoflurane *in vitro* before tumor injection, thereby limiting the anesthetic exposure to cancer cells and not immune cells or any other host cells. When applied to this *in vitro* data, the GEE showed significant main effect of time ($p < 0.001$), but no significant difference in main effect of isoflurane treatment ($p = 0.93$), main effect of gender ($p = 0.25$), time by

treatment interaction (p = 0.58), time by gender interaction (p = 0.26), treatment by gender interaction (0.29) and time by treatment by gender interaction (0.47). The lack of direct isoflurane effect on tumor growth shown in Figure 1D for the observed tumor sizes for male and female mice across all time points again indicates an immune system-dependent mechanism rather than a direct anesthetic effect on melanoma cells.

Discussion

Our observational studies demonstrate that isoflurane impacts melanoma growth in male mice only when an intact immune system is present, while no such effects on tumor growth occurred in wild-type, immune-competent females. A direct effect of isoflurane on tumor cells was proposed previously[7], but this was not apparent our melanoma model: tumor growth was not affected if the tumor was exposed to isoflurane before transplantation into wild-type males or females. The effect of inhalational anesthetics on tumor progression via its impact on the immune system has been studied and discussed in mice and humans[8]. Mouse models have previously demonstrated a detrimental effect of inhalational anesthetics on tumor spread[9,6]. Interestingly, published literature on the effect of anesthesia exclusively used male rodent models[9] or does not specify sex of mice[6], and the effect of sex in this process has not been reported. Differences in male and female immune functions are well established and our understanding of their clinical implications is expanding rapidly[4]. Little attention however has been directed to examine sex specific effects of anesthetics and how these relate to cancer progression. Our results indicate that male and female immune function may be affected differently by anesthetics, and further studies taking not only sex but also phases of the female estrous cycle into account are warranted. We do not suggest that human women are not affected by the detrimental effects of anesthesia on the immune system, and an effect of inhalational anesthetics on cancer growth in human women should not be excluded. In fact, human clinical studies examining the benefits of intravenous anesthesia vs. inhalational anesthesia found benefits in avoiding volatiles in both men and women, and additional studies describing the benefits of regional anesthesia demonstrate such effects in males as well as in females[10]. When studying the effects of volatile anesthesia on the immune system and their subsequent effect on cancer growth, especially in murine models, careful consideration should be given to the sex of the species being studied and comparative experiments are warranted when working with both rodent and human samples.

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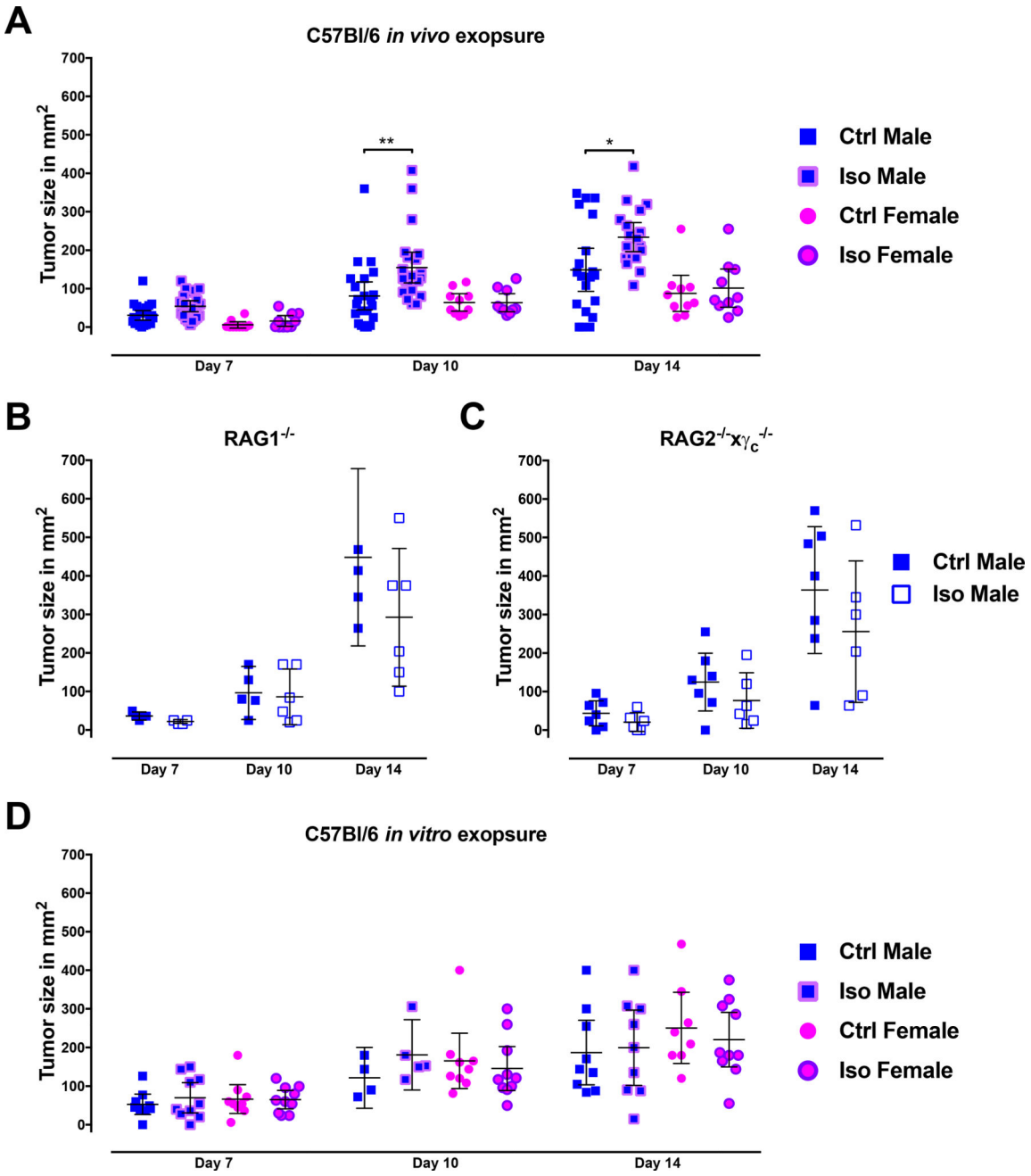

Figure 1. Murine melanoma growth is affected by isoflurane in a sex specific, immune-dependent manner

(A) Male mice show increased melanoma growth after *in vivo* exposure to isoflurane compared to female mice (Ctrl Male = 22/group; Ctrl Female n = 10/group, Iso Male n = 22/group, Iso Female n = 10/group). **(B,C)** The effect of isoflurane on melanoma growth in male mice was absent **(B)** in mice lacking functional B and T cells ($RAG1^{-/-}$) (Ctrl n = 5/group; Iso n = 6/group) as well as in **(C)** male mice lacking functional B, T and NK cells ($RAG2^{-/-}$ x $\gamma_c^{-/-}$)(Ctrl n = 7/group; Iso n = 4–6/group). **(D)** Furthermore, *in vitro* exposure of tumor cells to isoflurane prior to implantation did not lead to differences between female

and male mice (Ctrl Male = 4–9/group; Ctrl Female n = 9/group, Iso Male n = 5–10/group, Iso Female n = 10/group). In all experiments a significant effect of time confirmed overall tumor growth **(A – D)**. Data are presented as dot-plots for individual 'n' at each time point and shown as Mean and 95% Confidence Interval. Results were considered significant when $P \leq 0.05$. *: $p \leq 0.05$, **: $p \leq 0.01$.