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TOPICAL APPLICATION OF THYMIDINE DINUCLEOTIDE TO NEWBORN MICE REDUCES AND DELAYS DEVELOPMENT OF UV-INDUCED MELANOMAS

D.A. Goukassian[1], **A. Sharov**, **J. Rhodes**[2], **C. Coleman**[3], **M.S. Eller**[4], **T. Sharova**, **J. Bhawan**, and **B.A. Gilchrest**

Department of Dermatology, Boston University School of Medicine, Boston, Massachusetts, USA

To the Editor:

One major risk factor for cutaneous melanoma is ultraviolet (UV) exposure. Intense intermittent UV exposure and childhood sunburn are linked epidemiologically with melanoma risk, and in mice neonatal UV exposure promotes development of cutaneous melanoma (Noonan et al., 2001; Kannan et al., 2003). Other evidence that UV contributes to melanomagenesis includes increased risk for populations with extensive intense sun exposure, as well as for fair-skinned (Fitzpatrick's skin type I–II) individuals and patients with xeroderma pigmentosum, who repair photoproducts very poorly (Gilchrest et al., 1999; Kraemer et al., 1987).

We have previously shown that telomere homolog oligonucleotides (collectively called T-oligos), but not complementary or unrelated control oligonucleotides, have multiple anti-cancer effects (Eller et al., 1997; Puri et al., 2004; Gilchrest and Eller, 2009). T-oligo treatment prior to UV irradiation accelerates the removal of major UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4-photoproducts in cultured cells from newborn and adult donors (Goukassian et al., 2002), murine skin in vivo (Goukassian et al., 2004; Arad et al., 2008), and human skin ex-vivo (Arad et al., 2007). T-oligos have also been shown to cause cell cycle arrest, followed in many malignant cell types by apoptosis (Puri et al. 2004; Gilchrest and Eller, 2009). We have previously shown that treatment with T-oligos (specifically, thymidine dinucleotide - pTT) during chronic UV irradiation prevents development of SCC in hairless mice (Goukassian et al., 2004) and of BCC in Ptch-$1^{+/-}$ mice (Arad et al., 2008). In these models, intermittent topical pTT application enhances DNA repair of CPDs and 8-oxo-2′-deoxyguanosine, decreases mutagenesis, and in tumor

Corresponding author: Gilchrest, Barbara A., 609 Albany Street, J105, Boston, MA 02118, Phone: 617-638-5538, Fax: 617-638-5515, bgilchre@bu.edu.

[1]Current Address: Center of Cancer Systems Biology, Steward St. Elizabeth's Medical Center and Tufts University School of Medicine, Boston, MA 02135.

[2]Current Address: Stony Brook University Health Sciences Center School of Medicine, Stony Brook, NY 11794

[3]Current Address: University of New England, Portland Campus, 716 Stevens Ave, Portland, ME 04103

[4]Current Address: Department of Dermatology, University of Miami School of Medicine, Miami, FL 33136

Conflict of interest: Drs. Gilchrest and Eller are inventors on patents protecting the use of pTT in cancer treatment and prevention.

nodules increases apoptosis and decreases proliferation. pTT also strikingly reduces Cox-2 protein expression in UV-irradiated skin (Arad et al., 2008).

Many mutations associated with familial melanoma occur at the CDKN2A locus that encodes two distinct proteins, p16 INK4a and p14 ARF (p19 ARF in mice) (Chudnovsky et al., 2005). Several knockout (KO) and transgenic animal models have been developed to study p16- and p19-dependent molecular mechanisms of melanoma development. Of interest to modeling human melanomagenesis, when $p19^{ARF-/-}$ mice expressing H-ras driven by a tyrosinase promoter (Tyr-Hras/p19KO mice) are UV irradiated on day 2 or 3 after birth, there is a significant increase in melanoma development during early adulthood (Kannan et al., 2003). In this study we evaluated the effect of topical pTT treatment in this model.

Newborn mice were treated topically with a 100 μM solution of pTT (Midland Certified Reagent Company, Midland, TX) or the PG/DMSO vehicle alone on days 1 and 2, then UV irradiated on day 3 using FS40 sunlamps (10 mJ/cm^2) as metered at 285±5mm, an irradiation protocol known to cause melanomas by week 21 in approximately half the mice (Kannan et al., 2003).

pTT-treated mice began to develop melanomas during week 12, while vehicle-treated mice began to develop tumors during week 7; and by week 21, 71% vs 46% of the mice remained tumor free (Fig. 1A). All mice were examined weekly and sacrificed if their tumors were ≥1 cm in diameter or the animals appeared to be in distress. As well, in each treatment group, 4 mice died during weeks 15–17 for unclear reasons without evidence of tumor. Excluding these mice,at the end of 21 weeks, 13 of 24 vehicle-treated mice had tumors and 9 (69% of tumor-bearing mice) had to be sacrificed early, beginning at 11 weeks; but only 7 of the remaining 24 pTT-treated mice developed tumors and only one (14% of tumor-bearing mice) had to be sacrificed early, at 16 weeks (Fig. 1A, 1B and Table). Vehicle-treated mice had an average of 3 times as many tumors per mouse as pTT-treated mice at the end of 21 weeks ($p<0.01$, ANOVA post-hoc analysis) (Fig. 1C). Differences between the pTT and vehicle groups were significant beginning during week 12 and then each week through the end of the study as determined by SPSS multiple comparisons ($p=0.001$). At 21 weeks, vehicle–treated mice had an average tumor burden of 325±100.68 mm^3 vs.27.34±12.72 mm^3 ($p<0.001$) for pTT-treated mice (Fig. 1D), a >90% reduction.

Tumor size and multiplicity for the mice that were sacrificed or died early were included in the calculations as unchanged up to the termination of the experiment on week 21. Hence their contribution to total tumor burden, as well as possibly tumor multiplicity in these mice, is understated, as tumors would have been expected to continue growing. All tumors on surviving animals continued to grow throughout the study, and new tumors continued to appear. Most of the tumors were poorly-differentiated with a spindle-cell morphology; and all harvested tumors stained positively for Mart-1 (data not shown), confirming that they were indeed melanomas, despite being amelanotic in these albino mice.

Our data demonstrate that topical pTT application to newborn melanoma-prone mice only twice prior to UV irradiation delays and reduces subsequent melanoma development. This

effect is consistent with the previously documented ability of pTT to increase the rate and accuracy of UV-induced DNA damage repair when provided to cultured cells or to intact skin 24–48 hours prior to irradiation. One presumptive pTT target is the melanocytic stem cell that resides in the upper permanent portion of the hair follicle (Nishimura, 2011; Nishikawa-Torikai et al., 2011). These cells are understood to persist after the single irradiation on post-natal day 3 through multiple cycles of cell division and ultimately to give rise, weeks later, to a melanoma. Reducing the number of UV-induced melanocyte mutations, as shown to occur in pTT-treated epidermal keratinocytes in irradiated mouse skin (Goukassian et al., 2004; Arad et al., 2008), should logically reduce the number of Tyr-Hras/p19KO cells further predisposed to give rise to invasive melanoma. In addition, pTT effects on non-melanocytic cells may also have contributed to the >90% reduction in melanoma burden. For example, pTT treatment presumably reduced epidermal expression of Cox-2, the UV-inducible pro-inflammatory enzyme that enhances development of keratinocytic malignancies in irradiated mouse skin (Fischer et al., 1999; Pentland et al., 1999) and whose protein expression is markedly reduced over at least several days by pTT (Arad et al., 2008; Marwaha et al., 2005). Cox-2 inhibitors have been shown in intervention studies to reduce UV-induced non-melanoma skin cancers (Elmets et al., 2010) and more recently also melanomas (Curiel-Lewandrowski et al., 2011) in humans. Finally, pTT is known to have immunomodulatory effects (Cruz et al., 2000; Curiel-Lewandrowski et al., 2003) and may have modified interferon-mediated events recently shown to enhance melanomagenesis in mice following UV irradiation in the newborn period (Zaidi et al., 2011).

Of note, in human skin explants pTT treatment enhances epidermal pigmentation within 24 hours and this reduces initial UV-induced DNA damage by approximately 50% (Arad et al., 2007). This potentially additional photoprotective effect of pTT could not be observed in the albino mice used in these experiments.

Together, our findings further support a critical role for UV-induced damage in melanoma development and for photoprotection as a key preventive strategy.

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Abbreviations

BCC	Basal cell carcinoma
CPD	Cyclobutane pyramidine dimers
KO	Knock out
pTT	Thymine dinucleotides
PG/DMSO	propylene glycol 75% dimethyl sulfoxide 25%
SCC	Squamous cell carcinoma
T-oligo	telomere homolog oligonucleotides

UV Ultraviolet

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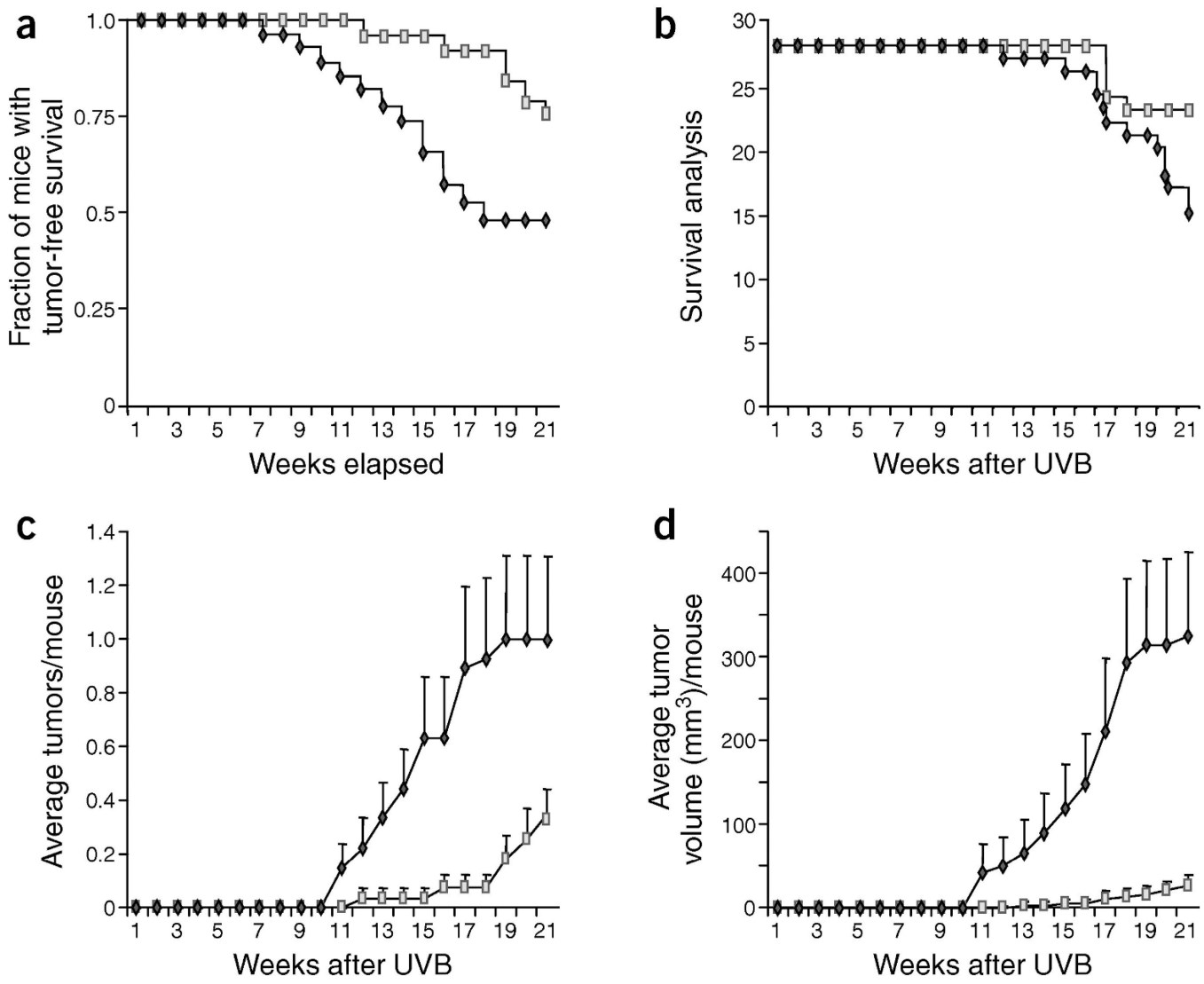

Figure 1.

Effect of pTT on melanomagenesis in UV-irradiated Tyr-Hras/p19KO mice over the 21 week study. Open symbols (rectangles): pTT-treated mice; black symbols (diamonds): vehicle-treated mice. **A**: Cumulative tumor free survival. Compared to vehicle, pTT markedly increased tumor-free survival ($p<0.04$, SPSS multiple comparisons). Black circles indicate one or more mice dying for unknown reasons or euthanized because of large tumors, after which they are censored from the analysis. **B:** Survival analysis. Four mice in each group died for unknown reasons in weeks 15–17. All other mice died or were euthanized because of large tumors. Compared to the vehicle, pTT increased overall survival ($p<0.04$, Kaplan Meier survival analysis) without affecting deaths from non-tumor related causes. **C**: Number of tumors per mouse (mean ± SD). Compared to the vehicle, pTT caused a marked decrease in the average number of tumors per mouse ($p<0.01$). **D:** Total tumor burden (mm^3) per mouse (mean ± SD), calculated using the formula volume=4/3 Pi

(length/2) (width/2)^2. Compared to vehicle, pTT caused a >90% reduction in average total tumor volume ($p = 0.001$).

Table

Fate of Tyr-Hras/ p19KO Mice

Treatment Group	Initial Number of Mice	Mice Dying or Euthanized By Week 21		Live Mice at Week 21	Mice with ≥ 1 Tumor During Study
		No Tumors	≥ 1 Tumor		
Vehicle	28	4	9	15	13
pTT	28	4	1	23	6