Inhibitory effects of viral infection on cancer development

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ABSTRACT

Immune responses evoked on viral infections prevent the dissemination of infection that otherwise leads to the development of diseases in host organisms. In the present study, we investigated whether viral infection influences tumorigenesis in cancer-bearing animals using a Drosophila model of cancer. Cancer was induced in the posterior part of wing imaginal discs through the simultaneous inhibition of apoptosis and cell-cycle checkpoints. The larvae and embryos of cancer-induced flies were infected with Drosophila C virus, a natural pathogen to Drosophila, and larval wing discs and adult wings were morphologically examined for cancer characteristics relative to uninfected controls. We found that viral infections brought about an approximately 30% reduction in the rate of cancer development in both wing discs and wings. These inhibitory effects were not observed when growth-defective virus was used to infect animals. These results indicate that productive viral infections repress tumorigenesis in Drosophila.

1. Introduction

The human body is equipped with immunity that prevents and mitigates various diseases. Once infected by microbial pathogens, the immune system is activated and induces changes in internal environments in the body through the production of soluble immune factors and emergence of immune cells. Therefore, it is reasonable to expect that microbial infections influence the development and progression of other diseases that the host organism suffers from beforehand. Although the effects of commensal microorganisms on the development, growth, and metastasis of tumors have been investigated to a certain extent as reviewed (Belkaid and Hand, 2014; Zitvogel et al., 2016, 2017, 2018; Roy and Trinchieri, 2017; Gallowa-Peña et al., 2017), limited information is currently available on the interaction between acute infections with microbial pathogens and tumorigenesis. This study was conducted to examine the effects of viral infection on the development and progression of cancer.

We used a Drosophila model of cancer, in which cell overgrowth was induced in specific cell types through the simultaneous inhibition of apoptosis and cell-cycle checkpoints (Dekanty et al., 2012). In this model, overgrown tissues continue to proliferate and metastasize when transplanted into healthy animals (Dekanty et al., 2012), indicative of neoplastic transformation. Drosophila develops cancer similar to humans (Vidal and Cagan, 2006; Rudrapatna et al., 2012; Gonzalez, 2013; Wang et al., 2014), and Drosophila cancer models have been used in research with the aim of discovering novel anticancer drugs (Dar et al., 2012; Willoughby et al., 2013; Markstein et al., 2014; Levine and Cagan, 2016) taking advantage of its small size and the applicability of genetic approaches. Furthermore, similarities in antiviral mechanisms have been reported between humans and Drosophila (Hughes et al., 2012; Jie and Cherry, 2014; Lamiable and Imler, 2014; Nainu et al., 2017). Therefore, we herein investigated whether viruses influence cancer after infecting cancer-bearing flies.

2. Results

2.1. Reproduction of Drosophila model of cancer

Dekanty and colleagues previously reported a procedure for the development of cancer in Drosophila (Dekanty et al., 2012). They simultaneously inhibited apoptosis and cell-cycle checkpoints by the ectopic expression of a gene coding for p35, a viral inhibitor of
Fig. 1. Cancer characteristics in wing imaginal discs. (A) (top) Bright field and fluorescence views of wing discs isolated from the third-instar wandering larvae of cancer-bearing and control normal flies. In fluorescence views, discs are outlined and the A/P boundary is indicated with artificial drawings. Cell overgrowth at the posterior part, the uneven expression of GFP among posterior cells, an altered A/P boundary, and morphological aberrations in the wing pouch (indicated with an arrowhead) are observed. Scale bars, 200 µm. (bottom) Four indicators of cancer induction are shown in percentage terms as the mean ± standard deviations after analyses of 25 discs in each of three independent experiments. Posterior part enlargement, ratio of the posterior portion to entire imaginal disc; Wing pouch disruption, rate of wing pouch with aberrant morphology; Uneven GFP expression, rate of posterior cells with uneven expression of GFP; Altered A/P boundary, rate of discs with altered boundary of anterior and posterior parts. (B) Alterations in the A/P boundary in the wing discs of cancer-bearing flies. Seven wing discs isolated from normal and cancer-bearing larvae were microscopically examined for alterations in the A/P boundary. Fluorescence views of the discs are shown as in (A). Scale bars, 200 µm.
caspases, and RNAi knockdown of genes involving checkpoints, respectively. This inhibition was applied to specific tissues using the GAL4-UAS system (Brand and Perrimon, 1993), in which the yeast-derived transcription factor GAL4 is expressed in a tissue- and/or developmental stage-dependent manner, and genes to be expressed possess a sequence named UAS, which is a binding site for GAL4. In this study, GAL4 was expressed under the transcription promoter of a gene coding for the caspase inhibitor p35, and a gene to express green fluorescence protein (GFP). As a result, the posterior half of wing discs labeled with GFP overgrew and exhibited cancer-like characteristics (Dekanty et al., 2012). We intended to reproduce this cancer model through the forced expression of p35 and RNAi knockdown of bub3, a checkpoint gene coding for a protein that inhibits the separation of duplicated chromosomes unless they properly attach to the spindle apparatus. Wing discs were isolated from the wandering larvae of cancer-induced and control normal flies, and examined by fluorescence stereo-microscopy. We observed several characteristics at high frequencies in the wing discs of cancer-induced flies: cell overgrowth at the posterior part, the uneven expression of GFP among posterior cells, an altered boundary at the anterior and posterior parts (A/P boundary), and the morphological disruption of the wing pouch (Fig. 1A, top panels). Cancer induction in this way seemed to have a little effect on the development of flies (data not shown).

We selected one characteristic, alterations in the A/P boundary, as an indication of the occurrence of cancer in wing discs because it was observed in most cancer-bearing animals (Fig. 1A, bottom panel) and easily identified. In the discs of normal non-cancer flies, the A/P boundary is initiated at the tip, curves down to the middle, then starts to protrude, falls down almost straight, and finally protrudes again reaching the bottom (Fig. 1A, top panels). In contrast, the wing discs of cancer-bearing flies do not follow this rule and have various patterns for the A/P boundary (Fig. 1B).

2.2. Productive infection of Drosophila larvae with DCV

We intended to infect embryos/larvae maintained in vials with Drosophila C virus (DCV), a non-enveloped positive-strand RNA virus and a natural pathogen to Drosophila, by directly adding viral suspensions to the surface of fly food containing developing embryos and larvae. In order to confirm a successful infection, RNA was prepared from larvae, which had been collected at specific time points after the addition of the virus, and analyzed by reverse transcription-mediated PCR (RT-PCR) using a primer set specific to the DCV genome. We designed primers such that a non-coding sequence intervenes to avoid the amplification of mRNA sequences. To ensure the successful preparation of cDNA from different RNA samples, a sequence corresponding to the ribosomal protein 49 (rp49) was also amplified as an unchanged internal control. A typical result after the separation of PCR products by gel electrophoresis is shown as Fig. 2A, and data from repeated experiments demonstrated that the copy number of the DCV genome increased after 72 h through to 144 h post-infection (Fig. 2B). This result indicated the growth of the virus in larvae and, thus, the successful infection of animals with DCV under these experimental conditions.

2.3. Inhibition of tumorigenesis by DCV infection

We then examined the effects of DCV infection on the development of cancer in wing discs. Cancer-bearing embryos/larvae were infected with DCV, and emerged third-instar wandering larvae were collected and dissected for the isolation of wing discs followed by a microscopic examination. The occurrence of cancer, which was confirmed by the observation of an altered A/P boundary, appeared to be reduced by about 30% after the infection with DCV (Fig. 3A) in a manner that was proportional to the initial burden of the virus (data not shown). To further confirm the inhibitory effects of DCV on cancer, we allowed larvae to develop into adults and examined their wings. Adult flies that had developed from cancer-bearing larvae exhibited an abnormal wing morphology, such as the emergence of wrinkles and cavities, and the loss of the vertical vein, which were all observed at a region corresponding to the posterior part of wing discs (Fig. 3B, left panels), indicating that these changes were due to cancer phenotypes in wing discs. DCV infection reduced the rate of these morphological changes in adult wings to a similar extent to that observed in wing discs (Fig. 3B, right panel). These results collectively indicated that the infection with DCV at the stage of embryos/larvae inhibited the development and/or progression of cancer in wing discs.

We then investigated whether this effect required the growth of the virus in flies. We prepared DCV defective in growth by exposing viral suspensions to UV light. After confirming the successful inactivation of the virus in an infection experiment using S2 cells as the host (data not
shown), the UV-inactivated virus was used to infect cancer-bearing embryos/larvae, and larval wing discs and adult wings were examined for morphological changes. The results obtained showed that UV-irradiated and, thus, growth-defective DCV did not exert inhibitory effects on cancer, as judged by either an altered A/P boundary in wing discs or abnormal morphologies in wings (Fig. 4). This result indicates the requirement of productive infection for DCV to inhibit the development/progression of cancer.

3. Discussion

The infection of cancer-bearing embryos/larvae with DCV inhibited cancer phenotypes in the wing imaginal discs and adult wings of Drosophila. This result suggested that viral infection has a negative effect on the development and/or progression of cancer, but the mechanisms underlying this action of DCV remain to be elucidated. In general, viral infections induce apoptosis in host cells that are subsequently eliminated by apoptosis-dependent phagocytosis (Nainu et al., 2017). However, cancer cells in wing discs are unlikely to be infected with DCV and removed by phagocytosis because wing discs are not included in a list of tissues for DCV infection; fat bodies and digestive tracts are the major targets (Chtarbanova et al., 2014). We previously reported that Drosophila phagocytes increase their phagocytic activity after the engulfment of apoptotic cells (Nonaka et al., 2017). Therefore, DCV infection may enhance the activity of phagocytes that have engulfed virus-infected cells undergoing apoptosis, and activated phagocytes might somehow attack cancer cells. Furthermore, other immune responses induced in Drosophila upon viral infection could play a role in the inhibition of cancer (Palmer et al., 2018).

Other modes of interplay between microbial infection and cancer have been reported. Infections with Gram-negative Pseudomonas aeruginosa may induce stem cell-mediated tumorigenesis in intestines.
through apoptosis in enterocytes of Drosophila (Apidianakis et al., 2009); immune responses to *P. aeruginosa* augment the dissemination of malignant cells in the hindgut of Drosophila (Bangi et al., 2012); and infections with influenza virus promote tumor growth at areas distant from the site of infection by depriving immune cells from cancerous tissues in mice (Kohlhapp et al., 2016). Although the context of these studies in terms of the type of infectious agents and tumors differ from that of ours, infections with microbes seem to favor cancer in these examples. Therefore, the effects of microbial infection on cancer appear to vary depending on the conditions of infections and cancer development. Further research is required in order to reach a general understanding of the involvement of acute infections with microbial pathogens in the development and progression of cancer.

In conclusion, the present study indicates that productive viral infections repress tumorigenesis in *Drosophila*.

### 4. Materials and methods

#### 4.1. Fly maintenance, cell culture, and virus stock preparation

All flies were maintained with standard cornmeal/agar media at 25°C. The following lines of *Drosophila* were used: *w^{118}, UAS-bub3-IR* on the 2nd chromosome (Vienna Drosophila Resource Center, Vienna, Austria; VDRC Transformant ID 21037) used to express double-stranded RNA for the RNAi knockdown of *bub3*, a cell-cycle checkpoint gene; *en-GAL4* on the 2nd chromosome (a gift from Dr. Shigeo Hayashi) used to express GAL4 at the posterior half of wing imaginal discs; *UAS-GFP* on the 2nd chromosome to express GFP; and *UAS-p35* on the 3rd chromosome (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN, USA: stock number 5073) used to express p35, a caspase inhibitor. S2 cells, a cell line established from *Drosophila* embryonic hemocytes, were maintained in cultures at 25°C and used as host cells for the propagation of DCV, as described previously (Nainu et al., 2015). To prepare virus stocks, S2 cell cultures were inoculated with an aliquot of DCV suspension and further maintained. Culture media were collected, titrated to obtain a 50% tissue culture-infective dose (TCID50), and kept frozen at −80°C as a stock, as described previously (Nainu et al., 2015). These stocks were used after a dilution with phosphate-buffered saline when necessary. Replication-defective DCV was prepared by exposing viral suspensions to UV light, as described previously (Nainu et al., 2015).

#### 4.2. Generation and analysis of a Drosophila model of cancer

The fly lines *UAS-bub3-IR; UAS-p35 and en-GAL4 UAS-GFP* (on the 2nd) were generated using the fly stocks described above. In each experiment, these flies were crossed to give rise to *UAS-bub3IR/en-GAL4 UAS-GFP; UAS-p35/+,* which was used as a cancer model. On the other hand, *w^{118}* was crossed with *en-GAL4 UAS-GFP* (on the 2nd), and the resulting *+/en-GAL4 UAS-GFP*; *+/+* was used as a non-cancer control fly. Third-instar wandering larvae were collected and dissected under a stereo-microscope to isolate wing imaginal discs. The isolated discs were examined by fluorescence stereo-microscopy (SZX16: Olympus, Tokyo, Japan), and images were captured using a camera (DP22: Olympus) and analyzed with an image analyzer (DP2-SAL: Olympus) using ImageJ (National Institute of Health, U. S. A.). To allow larvae to develop into adults, collected third-instar wandering larvae were transferred into new vials with fresh food and maintained for 6 days. One of the paired wings of emerging adult flies was collected for a morphological examination.

#### 4.3. Infection of flies with DCV

Two days after crossing for the generation of cancer-bearing flies, 0.4 ml of DCV suspensions (5.2 × 10^9 TCID50) were added to fly vials, which contained developing embryos and larvae, at the surface of fly food, and flies were further incubated at 25°C for 6 days until analyzed. With this protocol third-instar wandering larvae we collected for analysis had most probably been exposed to virus from early embryos. As a negative control, the same volume of culture supernatants of uninfected S2 cell cultures was used instead of viral suspensions, mock infections.

#### 4.4. Analysis of viral genome

The copy number of the RNA genome of DCV was semi-quantitatively assessed by RT-PCR. Approximately 30 larvae were homogenized with TRizol reagent (Thermo Fisher Scientific K.K., Tokyo, Japan), and total RNA was extracted using the acid-phenol method. Reverse transcription reactions were performed using the extracted RNA as a template, oligo(dN)6 as a primer, and ReverTraAce (Toyobo Co., Ltd., Osaka, Japan) as reverse transcriptase. The resulting cDNA was subjected to PCR using oligonucleotides, which contained the sequence of the DCV genome, as primers and Go Taq DNA Polymerase (Promega K.K., Tokyo, Japan). The mRNA of rp49 was similarly analyzed as an internal control. PCR products were separated on a 6% polyacrylamide gel and visualized by staining with ethidium bromide, and the intensity of each signal was digitized. The nucleotide sequences of the PCR primers were: 5′-GATGTGATCTGTGCTTCCATTATA-3′ (forward) and 5′-AAATGACAACCTAATCATATAAGA-3′ (reverse) for the DCV genome, and 5′-GACGCTTCAAGGGACACGATCTG-3′ (forward) and 5′-AAACGGTGTTCTGAATGA-3′ (reverse) for rp49 mRNA. The specificity of the primer used for a DCV genome analysis was confirmed by the analysis of RNA prepared from S2 cells that had been infected and mock-infect- ed with DCV (data not shown).

#### 4.5. Data processing and statistical analysis

Results from quantitative analyses were expressed as the mean ± standard deviations of data from at least three independent experiments. Statistical analyses were performed using Tukey’s test (for the data shown as Fig. 2B) and two-tailed Student’s *t*-test (for the data shown as Figs. 3 and 4). *P* values are shown in the corresponding figures or figure legends; any *p* values < 0.05 were considered to be significant.

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**Declarations of interest**

None.

**References**


