Research Article

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Stimulation of hepatocarcinogenesis by neutrophils upon induction of oncogenic *kras* expression in transgenic zebrafish

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Background & Aims: Chronic inflammation is a major etiological factor for hepatocellular carcinoma (HCC), but how immune cells respond in the initiation of hepatocarcinogenesis remains uncharacterized. This study aims to investigate the response and roles of neutrophils in early hepatocarcinogenesis.

Methods: By inducible expression of oncogenic *kras*^{V12} in hepatocytes in transgenic zebrafish combined with live imaging of neutrophils in transparent larvae, the response of neutrophils to oncogenic liver was characterized and their roles investigated by pharmaceutical and genetic manipulations.

Results: We found a rapid recruitment of neutrophils to the liver upon induction of *kras*^{V12} expression. Pharmaceutical stimulation of neutrophils resulted in further increases of neutrophils in oncogenic livers, liver size and tumor severity, while inhibition of neutrophils caused decreases of liver-associated neutrophils and liver size. Time-lapse video indicated that neutrophils had a stagnant migratory pattern meandering along the tumor edge but became relatively stationary upon entering the *kras*^{V12}-expressing liver. Both oncogenic hepatocytes and tumor-associated neutrophils (TANs) were isolated via fluorescence-activated cell sorting. Molecular analyses indicated a pro-inflammatory microenvironment, as marked by increased *tgfβ1a* expression in *kras*^{V12}-expressing hepatocytes and a loss of anti-tumor activities in TANs. Depletion of Tgf-β significantly reduced the number of TANs and the size of oncogenic liver.

Conclusions: An inflammatory cue from oncogenic hepatocytes upon induction of *kras*^{V12} expression causes a rapid recruitment of neutrophils to oncogenic liver and the neutrophils play a promoting role in early hepatocarcinogenesis.

Keywords: Liver; Tumor-associated neutrophil (TAN); Inflammation; Tgf-β; Tumor initiation.

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Abbreviations: HCC, hepatocellular carcinoma; FACS, fluorescence-activated cell sorting; Gcsfr, Granulocyte colony-stimulating factor receptor; TAN, tumor-associated neutrophil; NN, naïve neutrophil; RT-qPCR, reverse transcription-quantitative PCR; LPS, lipopolysaccharide; dpf, day post fertilization; H&E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Introduction

In the past decade, increasing evidence has indicated a dual role of neutrophils in a variety of tumors [1]. On one hand, activated neutrophils are capable of killing tumor cells through oxidative bursts [2] and secretion of anti-tumor cytokines such as TNF-α and IFNs [3]. On the other hand, in certain situations, neutrophils have also been found to promote tumor progression. Tumor-associated neutrophils (TANs) have long been observed to correlate to tumor progression in chronic colitis-associated carcinogenesis or gastric adenocarcinoma [4–6]. These alternatively behaved neutrophils are capable of releasing growth-stimulating signals, matrix-degrading proteases, and angiogenesis mediators [1,7], favoring tumor progression. Recently, it has been reported the existence of subtypes N1 (anti-tumoral) and N2 (pro-tumoral) neutrophils; the neutrophil plasticity appears to be regulated by transforming growth factor-beta (Tgf-β), which is often found to be secreted by cancer cells [4]. Neutrophils are induced by Tgf-β to acquire an N2 phenotype, which differs from N1 neutrophils that require inhibition of Tgf-β and sufficient ifn-β [4,8].

The influence between neutrophils and cancer cells are reciprocal. While tumor cells are capable of hyper-expressing pro-inflammatory molecules, mimicking the initial phase of wound healing [9], to attract neutrophils to the localized tumor microenvironment [10], the infiltrating neutrophils also have pro-angiogenic effects and promotes epithelial to mesenchymal transition during tumor progression [11,12]. This integral relationship between immune cells and tumor cells is particularly evident in HCC, which is a typical inflammation-associated cancer since the primary etiological factors, hepatitis B and C viruses, create an unresolved, chronic inflammation of the liver [13]. To date, systemic therapy has not been effective in HCC patients [14,15], although targeted therapy with a multi-kinase inhibitor, sorefenib, has limited efficacy in several clinical trials [16,17]. Thus, immune-based therapy could be a new promising approach for HCC patients. The study of the interaction between HCC and

neutrophils should provide much needed insights into the development of such a therapeutic approach.

Recently we have generated several inducible HCC models by transgenic expression of an oncogene in zebrafish hepatocytes [18–21]. A major advantage of these inducible models is the temporal control of cancer initiation to provide an excellent opportunity to characterize tumor initiation events, hitherto lacking in human clinical studies and other non-inducible tumor models. Furthermore, the transparency of zebrafish larvae allows us to monitor early *in vivo* hepatocarcinogenesis and progression, thus providing a plethora of opportunities to investigate the initiation events in hepatocarcinogenesis and the roles of various cancer hallmark factors in the process. In particular, we recently observed a prominent immune response in HCC progression and regression in one of our zebrafish HCC models based on RNA-Seq analyses [22]. In the present study, the interaction of neutrophils and oncogenic hepatocytes in hepatocarcinogenesis was investigated. We observed a rapid recruitment of neutrophils into oncogenic liver, which led to accelerated tumor progression. Molecular analyses of fluorescence-activated cell sorting (FACS)-isolated hepatocytes and TANs indicated changes of several important molecular pathways, including promotion of a pro-inflammatory microenvironment in oncogenic hepatocytes and decreases of anti-tumor in TANs. Thus, our data suggest a promoting role of neutrophils in early hepatocarcinogenesis.

Materials and methods

Zebrafish husbandry

Zebrafish were maintained in compliance with Institutional Animal Care and Use Committee guidelines, National University of Singapore. Four transgenic lines, *Tg(fabp10:rtTA2s-M2; TRE2:EGFP-kras^{G12V})* in a Tet-On system for inducible liver-specific expression of oncogenic *kras^{G12V}* [18], *Tg(lyz:DsRed2)^{nz50}* with DsRed-labeled neutrophils under the lysozyme C (*lyz*) promoter [23], *Tg(fabp10a:DsRed; ela3l:GFP)^{gz15}* with DsRed-labeled liver and GFP-labeled exocrine pancreas [24], *Tg(mpeg1:GFP)^{gl22}*, with GFP-labeled macrophages under the *mpeg*1 promoter [25], were used and referred to as *kras*, *lyz*, *fabp10*, and *mpeg*, respectively, in the present report.

Chemical treatment

20 μg/ml doxycycline (Sigma, D9891) was added from 3 days post-fertilization (dpf) to 8 dpf to induce *kras^{G12V}-EGFP* expression. Lipopolysaccharides (LPS) (Sigma, L4391), FPR-A14 (Tocris, 2826), PR-39 (Tocris, 1947) and SB431542 (Tocris, 1614) were first dissolved in dimethyl sulfoxide as stocks and used for larva exposure at 5 ng/ml, 2.5 μM, 50 nM and 2.5 μM respectively from 4 to 8 dpf. The dosages were selected based on the highest all-survival concentrations.

Statistical analysis

Statistical significance between two groups was evaluated by two-tailed unpaired Student *t* test using inStat version 5.0 for Windows (GraphPad, San Diego, CA). Statistical data are presented as mean value ± standard error of mean (SEM). Throughout the text, figures, and figure legends, the following terminology is used to denote statistical significance: $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001.

Other methods

Other methods are described in Supplementary Materials and methods, including morpholino knockdown and Tgf-β depletion; photography and image analysis; isolation of hepatocytes and neutrophils by FACS; RNA extraction, cDNA amplification and RT-qPCR (reverse transcription-quantitative PCR); and histological and cytological analyses.

Results

Rapid recruitment of neutrophils into kras^{V12}-expressing livers

To visualize the inflammation response, hemizygous *kras+* transgenic fish were crossed with *lyz+* homozygous fish for production of *kras+* and *kras−* offspring in the *lyz+* background, DsRed expressing neutrophils were monitored up to 96 hpi (hour post-induction with doxycycline). As shown in Fig. 1A and quantified in Fig. 1B–D, the total counts of neutrophils in the liver region was noticeably increased from as early as 8 hpi and became statistically significant from 16 hpi (Fig. 1B). These neutrophils within the vicinity of the liver, considered as TANs, were normalized against the liver size as neutrophil density. As shown in Fig. 1C, a significant increase in neutrophil density was observed from 8 hpi; thus, neutrophils were actively recruited to the site of tumor initiation within 8 hours of oncogene activation. In contrast, the increase of liver size became apparent only from 24 hpi (Fig. 1D), indicating that neutrophil recruitment preceded liver enlargement. To further evaluate the contribution of neutrophils to the increased liver size in *kras+* larvae, neutrophil density and liver size were plotted for each individual *kras+* larva and we observed a strong positive correlation (Pearson's coefficient, 0.62); in contrast, such a correlation was not present in *kras−* siblings (Pearson's coefficient, 0.20) (Fig. 1E).

Acceleration of hepatocarcinogenesis by stimulation of general immune response and neutrophils

To further demonstrate the role of inflammatory cells in initiation and progression of hapatocarcinogenesis, we first tested a general inflammatory stimulator, LPS, which has been demonstrated to stimulate the immune system in zebrafish larvae [26]. 5 ng/ml of LPS was used to treat zebrafish larvae from 4 dpf to 8 dpf. *kras+* larvae exposed to both LPS and doxycycline showed significant increases of both neutrophil count and density in the liver as compared to *kras+* larvae exposed to doxycycline alone and all *kras−* groups. Interestingly, there was also a further enlargement of liver size with the increased neutrophils (Fig. 2A). To investigate if the accelerated liver enlargement was indeed associated with increased neutrophil infiltration, FPR-A14, which is a formyl peptide receptor agonist and has been reported to potently activate neutrophils specifically *in vitro* [27], was used to challenge the *kras+* larvae from 4 dpf to 8 dpf. Liver neutrophil count and density in FPR-A14 and doxycycline double-treated *kras+* larvae were also significantly higher than those of their *kras+* sibling treated with only doxycycline and of all *kras−* groups (Fig. 2B), similar to that observed following LPS treatment. A further liver enlargement was also observed from these double-treated *kras+* larvae. To further demonstrate the effect of neutrophils, *kras+* transgenic larvae were also challenged with a neutrophil inhibitor, PR-39, a proline-rich anti-bacteria peptide that inhibits NADPH oxidase activity in neutrophils [28]. As shown in Fig. 2C, liver neutrophil count and density as well as liver size in *kras+* larvae exposed to PR-39 and doxycycline were all decreased as compared to *kras+* sibling controls treated with doxycycline alone and all *kras−* groups. Thus, there was a good correlation between numbers of infiltrated neutrophils and the size of oncogenic liver, suggesting an *in vivo* promoting role of neutrophils in early hepatocarcinogenesis.