

HYPOXIC INFLAMMATION, A DEADLY BRIDGE TO MALIGNANT TRANSFORMATION AND A FERTILE SOIL FOR CANCER PREVENTION

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Received 2013-06-12, Revised 2013-07-08; Accepted 2013-07-31

ABSTRACT

The interplay of the inflammatory microenvironment with its hypoxic niche and the potential mechanism by which they lead to malignant transformation has long been the subject of great controversy and continues to be an area of great interest today. In our previous studies we have examined this subject by using *Gia2* knock out mice as the focus of our research. These mice are well known for their tendency to develop chronic inflammation in the sub mucosa of their gut, with gradual worsening and development of colon adenocarcinoma in most as they age. It has also attracted our attention that they develop a significant increase in the number of hypoxic niches in their sub mucosa, proven by EF5 staining. In contradistinction to MSI-high colon adenocarcinomas, we have also shown that histone deacetylation rather than MLH1 promoter methylation is the main mechanism of MLH1 and MSH2 deactivation in these mice. Here we show that hypoxic niches evolve under massive selective pressure of the inflammatory microenvironment as a protective shield offering survival advantage by the up regulation of NFκB and its downstream pathways, which indeed independent of true hypoxia leads to stabilization of HIF as well, securing a dual mechanism for perpetuation and expansion of hypoxic niches. We incorporated western blot and Luciferase assay of cells exposed to hypoxia+/-inflammatory cytokines to acquire data.

Keywords: Hypoxia, Inflammation, Cancer Prevention

1. INTRODUCTION

Despite the fact that it has been known for a long time that chronic inflammation in the mucosa and sub mucosa of patients with inflammatory bowel disease, as well as mouse models such as *Gia2* KO mouse act as harbingers for malignant transformation, until this writing, no one has been able to connect the pieces together and describe the mechanism by which malignancy arises in real life IBD patients and *Gia2* KO mouse (Triantafillidis, 2009; O'Connor *et al.*, 2010; Westbrook *et al.*, 2010).

In patients who have been diagnosed with ulcerative colitis, prophylactic total colectomy, which is associated with significant morbidity and close surveillance is currently the mainstay of approach (Ma *et al.*, 2012; Sandborn *et al.*, 2009; Samuel *et al.*, 2013). Deep understanding of the mechanism of malignant transformation might enable us to develop a much better prophylactic therapy for such patients either by reversing chronic inflammation or by breaking the bridge between chronic inflammation and malignant phenotype. Furthermore, patients that have been diagnosed with Crohn's disease undergo remitting,

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relapsing cycles of inflammation of different severity and duration as well and usually run a devastating natural history with variable responses to anti-inflammatory and immunosuppressive agents (Colombel *et al.*, 2010; Smith *et al.*, 2009; Strober *et al.*, 2010). Despite the fact that there are many different theories, ranging from genetic to environmental factors, these diseases have remained incurable thus far (Benchimol *et al.*, 2011; Gleeson *et al.*, 2011).

2. MATERIALS AND METHODS

2.1. Luciferase Assay

T293 cells were transfected with a CMV vector Ranilla, expressing NF κ B by using conventional transfection methodology. The cells were then grown in a 10cm culture dish and placed in a hypoxic chamber as well as conventional incubators with and without TNF and LPS at different concentrations and for different periods of time of up to 48 h. NF κ B expression was also measured using Luciferase assay.

2.2. Protein Expression

Cells were lysed according to standard protocols using protein lysis buffer to obtain protein and western blot was conducted using a SDS-page gel electrophoresis system.

2.3. Mrna Expression

Total RNA was extracted according to standard protocols and reverse transcribed to DNA from HT29 and CCIC spheroids which have been exposed to normoxic and 1% hypoxic conditions.

2.4. Spheroid Formation

By using standard stem cell medium containing EGF, b-FGF, N2 and B27, in ultra low attachment flasks, spheroids were generated out of CCIC and HT29 cells and their stemness verified using stem cell markers.

3. RESULTS

Chronic inflammation in the sub mucosa of our Gia2 KO mouse model leads to a significant increase in hypoxic niches, shown very clearly by EF5 immuno fluorescent staining (Marotta *et al.*, 2011).

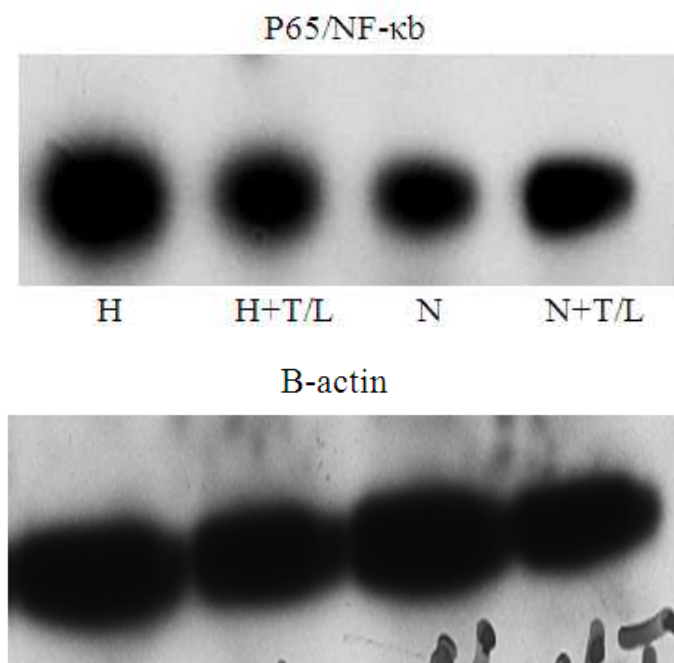


Fig. 1. p65 expression in Colon Cancer Initiating (CCIC) cells show a greater density under Hypoxic (H) conditions with TNF α and LPS (T/L) with a lower density under Normoxic (N) conditions with the same variables. B-actin was used as a loading control and confirms the integrity of protein loading

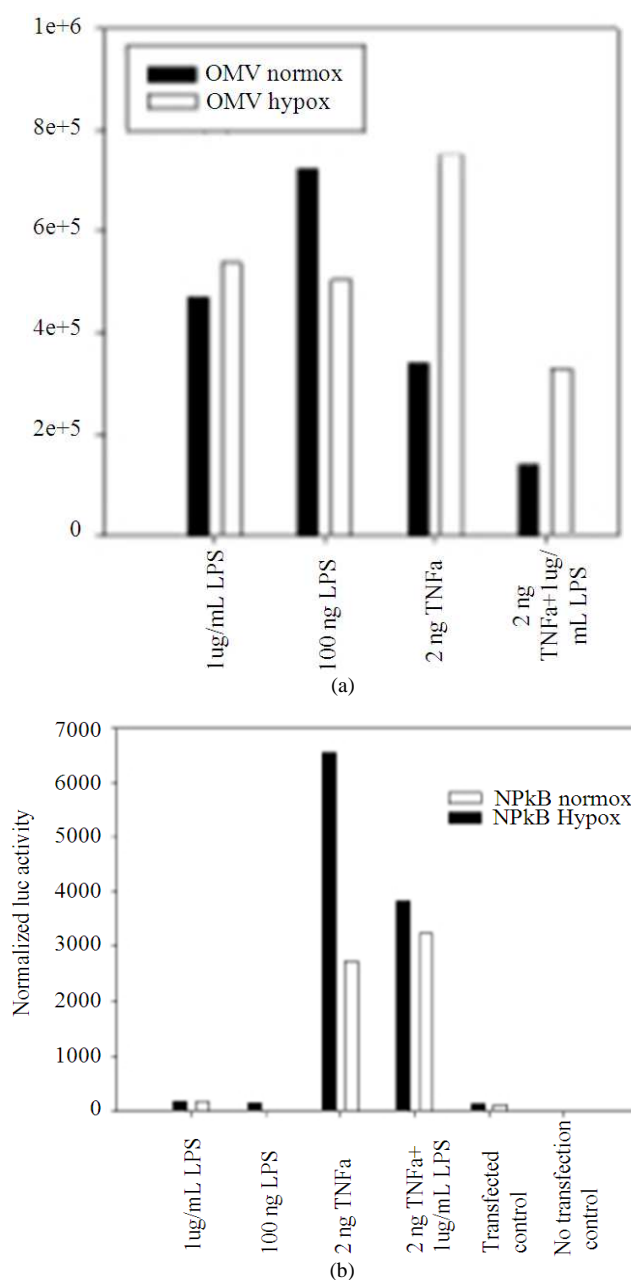


Fig. 2. The luciferase assay using the T293 cell line for transfection helps confirm our findings, demonstrating a significant upregulation of NFκb under hypoxic conditions relative to normal

In our *in vitro* experiments, exposure of different cell lines (T293, HT29, CCIC), to inflammatory cytokines such as TNF and LPS, with and without hypoxia led to up regulation of HIF1α (**Fig. 3**) and its stabilization by over expression of NFKb, as shown by western blot (**Fig. 1**). Hypoxia alone acted as an

independent and stabilizing factor as well, which is also shown in our raw data (**Table 1**) and further supported by our luciferase assay (**Fig. 2**). To cross-examine our data, we used the HT29 cell line under similar conditions and we obtained similar results (**Fig. 4**).

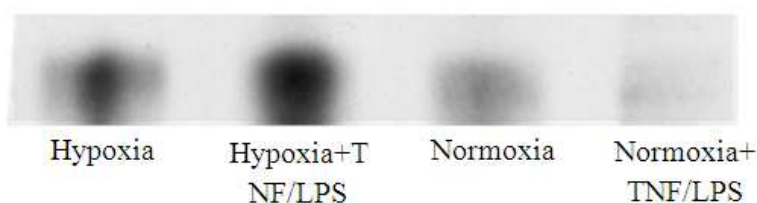


Fig. 3. CCIC exposed to hypoxia+/-TNF and LPS Vs their Normoxic counterpart clearly shows upregulation of under hypoxic conditions, which increases following treatment With TNE and LPS

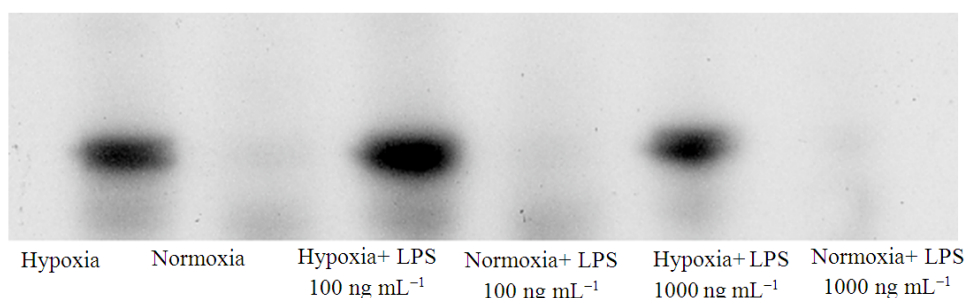


Fig. 4. p65 expression in HT29 cell line demonstrating significant overexpression in hypoxia with LPS at 100/1000 ng/ML of medium compared to normoxia under similar conditions

Table 1. Based on the raw data presented using T293 cell line, a significant change is clearly evident with TNF α under hypoxic conditions with no significant difference with LPS. NFkB expression correlates more closely with TNF α and incrementally so with increased concentration

	LARII	S and G	Ratio
Hypox 48 hrs alone	321.600	2.526	127.32
Then hypox 48+10 ng mL ⁻¹ LPS	1430.000	4.644	307.92
Then hypox 48+100 ng mL ⁻¹ LPS	1080.000	4.033	267.79
Then hypox 48+2 ng mL ⁻¹ TNF α	3009.000	4.988	603.25
Then hypox 48+10 ng mL ⁻¹ TNF α	1751.000	0.992	1765.12
Hypoxia 48 hrs no transfection control	0.058	0.250	0.23
Normoxia 48 hrs alone	1737.000	12.830	135.39
Then normoxia 48+10 ng mL ⁻¹ LPS	448.000	1.418	315.94
Then normoxia 48+100 ng mL ⁻¹ LPS	2120.000	9.705	218.44
Then normoxia 48+2 ng mL ⁻¹ TNF α	1599.000	4.399	363.49
Then normoxia 48+10 ng mL ⁻¹ TNF α	1206.000	1.573	766.69
Normoxiaia 48 hrs no transfection control	0.020	0.270	0.07

Hypoxia acted independently and synergistically with TNF and/or LPS, as far as over expression of NFkB is concerned, thus securing a dual mechanism of survival under harsh inflammatory micro environmental stimuli.

4. DISCUSSION

Despite the fact that chronic inflammation in different organs has been found to be associated with malignant transformation, ranging from adnexal/lacrimal gland maltomas in response to

Chlamydia Trachomati (Kram *et al.*, 2010) to thyroid lymphoma of Hashimoto's thyroiditis (Rapoport and McLachlan, 2012) gastric maltoma secondary to Helicobacter Pylori (Pervez *et al.*, 2011), to hepatocellular carcinoma secondary to chronic hepatitis B and C (El-Serag, 2012; Lok *et al.*, 2009), colon adenocarcinoma due to ulcerative colitis (Jess *et al.*, 2012), EBV and Papilloma virus related Burkitt's lymphoma and squamous cell carcinoma of head and neck as well as cervix (Lajer *et al.*, 2012; Guan *et al.*, 2012) and most recently implications of chronic

inflammation in some leukemias (Chen *et al.*, 2010), until this writing no one has shown the detailed dynamics and interplay of chronic inflammation with malignant transformation. Here we clearly show that chronic inflammation triggers the formation and expansion of hypoxic niches, which come into existence as a protective shield in response to the high selective pressure generated by harsh inflammatory microenvironment. We further define that convergence of hypoxia and chronic inflammation would offer the dual survival advantage to these cells by upregulation of NF κ B and stabilization of hypoxic niches through up regulation of HIF in a synergistic fashion. It also been widely demonstrated that the activation of pathways such as FOXO3a would add further to the preservation of the hypoxic niche, which would become the nest for nurturing cells with stemness capabilities, supported by spheroid formation and stem cell marker expression (Bakker *et al.*, 2007). Clearly the probability of malignant transformation would increase dramatically in these massively expanded hypoxic niches, through random genetic events, as well as the direct and indirect effects of exposure to environmental carcinogens.

Our model allows for early/pre-emptive intervention with either anti-inflammatory agents and perhaps by recruiting genetically engineered cells into either the inflammatory microenvironment or hypoxic niches to generate anti-inflammatory proteins or oxygen respectively.

This model also allows a new scoring system for measurement of success of our new approach to these old problems by serial biopsies and staining for either the markers of hypoxia, EF5 or inflammation by immunostaining for TNF or LPS.

As of today our biomedical society has learned that eradication of *H. pylori* could lead to reversal and prevention of gastric maltomas, eradication of hepatitis B and C would lead to prevention of hepatocellular carcinoma, vaccination against the prevalent serotypes of papilloma virus could lead to prevention of squamous cell carcinoma of cervix. In the near future we should be able to send our genetically engineered cells by using nanodelivery mechanisms to the birthplace of malignancy, namely the inflammatory microenvironment and hypoxic niches on a selective basis.

5. CONCLUSION

We have discovered that the generation and expansion of hypoxic niches in chronic inflammatory

conditions is a response to the deadly inflammatory microenvironment. This “protective shield” ultimately nurtures cells with stemness capabilities and promotes malignant transformation. Furthermore, our findings could generate a clinical platform for a future scoring system that could be used for the assessment of novel preventive and therapeutic measures.

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