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Research Article

Linking Cancer to Metabolic Syndrome through it's Individual Contributory Components: Oxidative Stress and Renin-Angiotensin-Aldosterone Axis Dysregulation

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ABSTRACT

Impaired signaling pathways as a result of the increased oxidative stress and general metabolic dysregulation seen in metabolic syndrome may contribute to the increased cancer risk seen in otherwise healthy patients; and subsequent development of progressive cancer characterized by metastatic process. Oxidative stress leading to insulin resistance is a major component of the metabolic syndrome. Dysregulation of the Renin-Angiotensin-Aldosterone (RAAS) axis is known to be one of the major risk factors for metabolic syndrome; and recently has been shown to play a significant contributory role in the development of oxidative stress; leading up to insulin resistance; in the metabolic syndrome by several different groups. In recent years; angiotensin and aldosterone have emerged as important contributory pathogenic factors for the development of metabolic syndrome; primarily through their ability to cause cellular oxidative stress. Angiotensin (Ang II) can also induce oxidative stress through activation of NADPH oxidase. Recently; metabolic syndrome has been associated with increased risk for a variety of cancers. Since oxidative stress is a critical component of the metabolic syndrome; it stands to reason that Ang II excess would increase oxidative stress and contribute further to increased cancer risk in terms of epigenetic dysregulation in stressed cells. Thus the purpose of this study is to check the effect of the RAAS axis on cancer development and characterize the intracellular signaling pathways involved. Specifically; the correlation of cancer incidence and spread to Ang II treatment and levels of oxidative stress will allow us to analyze the role of the vital contributory components of the metabolic syndrome in cancer.

Keywords: Cancer; Metabolic syndrome; Oxidative stress; Angiotensin 2; Aldosterone; Reactive oxygen species; Obesity; Diabetes; Insulin signaling; Cardiovascular disease

RESEARCH PLAN

Background and significance

Metabolic syndrome is a recognized clinical entity which can persist for years and precedes the development of overt clinical conditions [1]. It is characterized by very mild laboratory and functional disturbances characterized by impaired metabolic signaling and disordered cellular homeostasis [2]. Oxidative stress and mild but persistent inflammation are the main factors leading up to the development and progression of metabolic syndrome [1-3]. In addition, metabolic syndrome is characterized by the feature of insulin resistance thereby leading to reduced glucose uptake [1,3,4]. The Renin-Angiotensin-Aldosterone Axis (RAAS) is frequently implicated in the pathogenesis of the metabolic syndrome [3-6]. Ang II, which is one of the main effector molecules of the RAAS axis, can bind the NADPH oxidase receptor directly and can cause oxidative stress [7,8]. In addition, it can also cause increased intracellular oxidative stress through activation of its own receptor AT1, which leads to stimulation of the stress-responsive kinases and increased production of intracellular ROS [9]. All of this can stimulate activation of pro-inflammatory pathways and cell necrosis, contributing eventually to epigenetic dysregulation of cellular homeostasis and cancer development. Since both oxidative stress and RAAS axis dysregulation can contribute to cellular injury [5], it would be interesting to elucidate the role of these components in the development of cancer in the context of the metabolic syndrome.

Metabolic syndrome can be stimulated *in vivo* upon treating

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healthy cells or animals with high doses of Ang II for prolonged periods [10]. Elucidating the signaling pathways leading up to metabolic dysregulation seen under these conditions would not only help to characterize the precise mechanism by which RAAS contributes to cancer in response to oxidant stress but may also serve to identify the mechanisms of cancer development in the context of the metabolic syndrome.

Hypothesis

The overall purpose of this research project is to find out whether the RAAS component Ang II is able to stimulate the oxidative-stress mediated development or progression of cancer process.

In this regard, the following 2 aims can be proposed:

Aim 1: To determine if Ang II causes or contributes to dysregulated cellular homeostasis in the context of the metabolic syndrome, and to characterize the pathways involved.

Aim 2: To measure the effect of Ang II excess on downstream inflammatory and necrotic processes, oxidative stress levels and overall cell survival/turnover which will provide an indirect measure of the extent of epigenetic dysregulation in this model.

Overall strategy

In order to approach this project, murine models of metabolic syndrome and cultured cells will be subjected to chronic Ang II exposure with or without pre-treatment with an AT1 receptor specific blocker such as olmesartan, after which the tissue or cells will be collected and subjected to SDS-PAGE and immunoblotting analysis to study the expression of marker proteins or signaling molecules, known to contribute to the pathogenesis of cancer. Activation of stress-responsive kinase pathways or inflammatory pathways can be evaluated by probing for their protein content and phosphorylation status as well as those of downstream substrates using antibodies. Antibodies to stress-sensitive MAPKs such as p38 and JNK are readily available, as are antibodies to growth/survival promoting molecules ERK1/2 and AKT and the inflammatory NF-kB. In addition, ROS assays can be used to measure the levels of oxidative stress, helping to differentiate between oxidative stress-dependent and independent signaling pathways. Combining the data obtained from the immunoblotting studies and ROS assays with measures to quantify cell survival/turnover such as cells counts, TUNEL staining for apoptosis, Trypan Blue evaluation in culture, and other similar methods will serve to correlate the signaling data with cellular damage and provide a framework for characterizing the role of Ang II and oxidative stress in cancer development in the context of the metabolic syndrome.

RESEARCH DESIGN AND METHODOLOGY

Research design

Aim 1: Mouse models of metabolic syndrome (such as ob-/ob- mice) characterized by obesity and insulin resistance due to deficient leptin signaling are readily available from the Jackson laboratory [11,12]. This mutation on a C57BL/6J background (C57BL/6 mice used as control) produces insulin resistance and other features of the metabolic syndrome but does not result in overt diabetes [11]. Also, cell-lines for cultures are available commercially, as also some human cell culture models have been characterized, which could be used for correlation studies [13]. In addition, primary cultures could be generated from the mouse models [14]. Infusing the C57BL/6J ob/ob mice with a slow pressor dose of Ang II at 0.5 mg/kg/day results in acceleration of the metabolic syndrome phenotype with the characteristic features of insulin resistance, oxidative stress, RAAS dysregulation and impaired liver metabolic signaling [10,15]. Similarly, treating cultured hepatocytes with 100 nM Ang II for 24 hours (chronic exposure) results in a similar phenotype [10]. Incubating the hepatocytes in starvation medium for several hours prior to treatments results in elevated stress conditions and primes the response to Ang II treatment [10]. Prior treatments with olmesartan, an AT1 receptor-specific blocker, in animals (0.5 mg/kg/day) or in culture (1 μM) have been shown to successful block Ang II-mediated effects and downstream signaling [10,15]. Evaluating the protein content and phosphorylation patterns of stress-responsive kinases and other marker molecules known to precede the development of cancer by immunoblotting will provide a measure of the cellular epigenetic dysregulation in response to Ang II treatments in the context of oxidative stress and metabolic syndrome.

Aim 2: For addressing Aim 2, stated above, after subjecting the animals and cells to their respective pre-established treatment periods, animal tissue or cells respectively will be analyzed by ROS assays or immunoblotting studies. Antibodies to NADPH oxidase subunits as well as to signaling pathway molecules and stress-responsive kinases mentioned earlier will be used. The ROS assays will serve to determine if the levels of oxidative stress increase further after Ang II introduction. In addition, they will help to determine if some of the downstream signaling pathways induced by Ang II may be oxidative stress dependent. Finally, TUNEL staining of individual cells and Trypan Blue analysis of cells in culture will serve to establish indirect measures of cell survival/turnover and determine if Ang II causes/contributes to cancer through oxidative-stress induced apoptosis/necrosis mechanisms.

Methodology and explanation of techniques

Immunoblotting: Liver tissue from animals or cultured cells after Ang II exposure, with or without prior treatment with AT1 receptor specific blocker olmesartan, as mentioned above, will be collected, subject to non-denaturing lysis and protein obtained as per standard protocols. The phosphorylation patterns and protein content of the proteins mentioned above will be readily tested by standard immunoblotting techniques, after equalizing the amount of protein as mentioned above, using readily available commercial antibodies raised against these proteins. Beta-actin or other similar commercially available probes will be used for loading controls. The protein content and phosphorylation data will be analyzed using standard protein quantitation software.

ROS assays: For ROS assays, tissue collected from Ang II treated animals, or intact cells in culture, will be washed with warm buffer and used immediately in the assay, as per manufacturer's protocol.

POTENTIAL RESULTS

It is expected that oxidative stress will be significantly elevated due to Ang II treatment in conditions of metabolic syndrome and contribute to the development and progression of epigenetic

cellular dysregulation, which precedes cancer. In addition, treatment with the AT1 receptor-specific blocker olmesartan will serve to characterize the particular signaling networks involved in the response to oxidative stress and will show whether the pathways are oxidative stress-dependent or independent. In addition to generating significant information pertaining to the signaling networks involved in the link between Ang II and cancer in response to oxidative stress, the study will also provide additional translational and research value for cancer progression and risk in the context of the metabolic syndrome and insulin resistance, as it will serve to identify the regulatory and/or signaling pathways involved in RAAS-mediated impaired metabolic signaling pathways. In all, it may generate a wealth of data useful for potential future studies.

PITFALLS AND ALTERNATIVE STRATEGIES

If the proposed dose or time of Ang II treatment in the model of metabolic syndrome does not lead to the desired cellular dysfunctional response, alternative doses or treatment periods will be evaluated in order to achieve the optimal response in terms of increased oxidative stress and cellular dysregulation. In case of a discrepancy between the animal and cell culture models, the data will be subjected to detailed analysis and additional experiments conducted to confirm the data one way or the other. If the increased expression of Ang II does not generate additional oxidative stress, in addition to that already present due to the dysregulation caused by the metabolic syndrome, additional molecules or pathways will be explored to elucidate whether the role of Ang II in cancer development and progression is independent of oxidative stress or whether Ang II contributes to the oxidative stress indirectly by stimulating or activating these other molecules or pathways.

SUMMARY AND FUTURE DIRECTIONS

In summary, we anticipate that this research study will serve as an important study in helping to develop and characterize the link between cancer and cellular dysregulation in the context of increased oxidative stress, insulin resistance and RAAS dysregulation, which are all characteristic features of the clinically recognized metabolic syndrome, and will provide future direction and starting points for initiating and pursuing new projects. In addition, the research project may suffice to establish Ang II as a novel biomarker for cancer risk in metabolic syndrome, or as a potential drug target candidate for future studies.

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