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13. ABSTRACT (Maximum 200 Words) Cancer cells can generate constitutively reactive oxygen species (ROS), which are thought to promote cell proliferation, cell motility, invasion and angiogenesis, all of them prerequisites for tumor metastasis. Recently novel ROS-generating enzymes termed Nox have been identified in epithelial cells. Transfer of Nox into non-transformed fibroblasts increased ROS production and rendered these cells tumorigenic. Our project will identify Nox family members in cancer cells and evaluate their regulation and cellular function. Breast cancer cell lines were screened by RT-PCR for the presence of various <i>nox</i> and neutrophil NADPH oxidase genes and contained Nox3, Nox4, Nox5 as well as p22 ^{phox} and p67 ^{phox} . Preliminary experiments show that the regulation of Nox-based enzyme systems may be under control of p22 ^{phox} , growth factors and integrins. We observed down-regulation of ROS production when attachment of cells was abolished or under serum withdrawal. In terms of function, Nox4-stimulated ROS generation led to up-regulation of VEGF, a promoter of angiogenesis. The activity of deregulated Nox proteins in cancer cells may have wide ranging implications in tumorigenic events including metastasis.				
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INTRODUCTION:

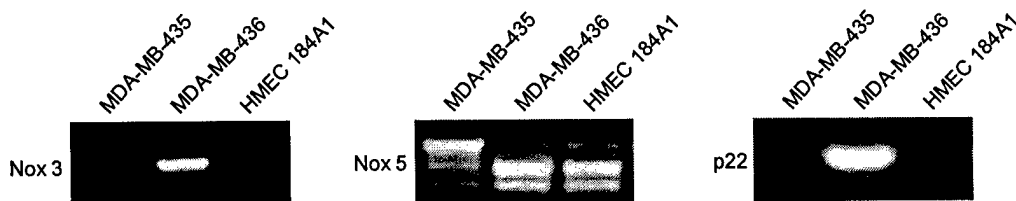
Certain human carcinoma cell lines produce reactive oxygen species (ROS) constitutively. This affords them potentially with an advantage in cell proliferation, and in migration and invasion of surrounding tissues by degrading the surrounding extracellular matrix and by increasing their motility. The basis for generation or upregulation of ROS in cancer cells is so far unknown. We hypothesized that recently identified, novel ROS-producing enzyme systems may be involved in this phenomenon and proposed to define members of this family in breast cancer cells and to evaluate their role in events leading to metastasis.

BODY:

To provide more continuity for this report, we decided to change the order in which Technical Objectives and their results to date are presented. All three objectives have been investigated in parallel and their outcomes depend on each other.

Objective 3: Characterization of the ROS generating enzyme in cancer cells

We have spent considerable time and effort in analyzing the identity of the ROS-generating enzyme in cancer cells. According to our hypothesis, we focused our efforts on NADPH oxidase components and cytochrome b₅₅₈ homologs. We reported before that *nox2*, the NADPH oxidase subunit of cytochrome b₅₅₈ (formerly gp91^{phox}) and *nox1* were not present in breast cancer cells. We designed primers for RT-PCR to detect *nox3*, and the newly discovered homologs *nox4* and *nox5* (1-5, 7). While almost all cancer cells contained low levels of *nox4*, only the breast cancer cell line MDA-MB 436 expressed *nox3* message (Figure 1). Several primer sets to detect *nox5* were designed. RT-PCR with these primers determined that the originally published sequence of a *nox5* isoform containing N-terminal EF hands was not present in any cell line tested. A different set of primers was designed to determine if *nox5* lacking the EF hands was present. This *nox5* isoform is more homologous to the previously described *nox4* and *nox1*. Figure 2 shows that *nox5* short form was detected in two of the breast cancer cell lines and in the immortalized breast epithelial cell line HMEC184. We observed under various PCR conditions multiple bands which were amplified and sequenced. All three major bands represented either full-length or fragments of *nox5*. Since the database lists six slightly different *nox5* genes we assume that these cell lines contain various forms of *nox5*. We will design another set of *nox5* short form primers to clarify these results.



RT-PCR for *nox 3*, *nox 5* and *p22^{phox}* message in breast epithelial and breast cancer cell lines.

Breast cancer cell lines were also tested for the presence of components of the neutrophil NADPH oxidase which form the active electron transfer complex in these cells. While we did

not detect $p47^{phox}$ or $p40^{phox}$, almost all of the cell lines showed high levels of $p22^{phox}$. This was expected since immunoblots had demonstrated that $p22^{phox}$ is ubiquitously expressed in mammalian cells. It is very interesting in this respect that the only two cell lines without $p22^{phox}$ in our screening are the breast epithelial cell line HMEC184 and MDA-MB 435 (Figure 3). This has been confirmed by Western blot with specific anti- $p22^{phox}$ antibodies. MDA-MB 436 contain not only $p22^{phox}$, but also as the only cell line the NADPH oxidase component $p67^{phox}$ (not shown). We performed also additional immunoblotting experiments to detect Nox proteins in cancer cell lysates. Only two anti-Nox antibodies have been prepared so far, one directed against Nox1 (by D. Lambeth, our collaborator) and one against Nox4 (by H. Sumimoto). The Nox1 antibody is not sensitive enough to detect endogenous Nox1. We were not able to obtain the anti-Nox4 antibody from Japan. We tested several anti-Nox2 antibodies, which have been characterized in neutrophils. Unfortunately, none of these antibodies cross-reacted with endogenous or recombinant Nox isoforms (up to 250 μ g total protein loaded).

We conclude from these experiments that breast cancer cells contain Nox homologs and various components of the NADPH oxidase, but that their distribution and their ability to produce reactive oxygen species does not correlate directly with the presence of these proteins. It is obvious that we do not understand at this moment how Nox proteins are regulated and which Nox proteins are indicative of constitutive ROS generation. Cell-free assay systems reconstituted of cancer and normal epithelial cell fractions showed that ROS generation was independent of GTP γ S or of neutrophil oxidase activators. We have therefore decided to focus on the two most abundant Nox isoforms (Nox4 and Nox5), since they seem to display a very different behavior when transfected into cells and to elucidate at first their function and regulation. These efforts may provide us with the necessary information to investigate then the regulation and function of Nox isoforms in cancer cells.

Objective 2: Identification of signals regulating ROS production in cancer cells

We investigated which intracellular signaling pathways or extracellular cues might enable cancer cells to produce ROS constitutively. Dominant negative as well as constitutively active signaling molecules especially GTPases were introduced into cancer cells. The GTPase Rap1A was selected since Rap1A can form a complex with neutrophil Nox2 and affects attachment of cells. The infection of cells with various Rap1A constructs showed no changes in ROS production. The only signaling molecules which decreased ROS production to approximately 50% were Rac1 and Ras mutants. Starvation of cancer cells for 24-48 h suppressed ROS generation, which could be re-stimulated when starved cells were exposed to fresh serum (Figure 4). After 48 h the levels of ROS production reached the levels observed before starvation. This observation suggests that an autocrine loop or feedback mechanism exists which may be dependent on regulatory growth factors or hormones.

Since the identity of the ROS-generating enzyme in breast cancer cells is still under investigation, and the regulation of numerous Nox isoforms is unclear, we decided to evaluate Nox regulation in a more defined system than cancer cells with their multiple genetic changes. We prepared various cell lines stably expressing Nox4 and started to investigate Nox4 and Nox5 regulation by transient expression in non-transformed cell lines (HEK293, Cos). We have cDNAs for Nox1, Nox4 and Nox5 (short

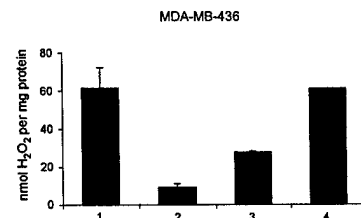


Fig. 4: ROS generation of MDA-MB 436 cells after 48h in 10% serum (1), 48h no serum (2), 48h serum starved plus 24h 10% serum (3), 48h serum starved plus 48h 10% serum (4)

form) available which were cloned into expression vectors containing epitope tags. Epitope tags will allow us to ensure Nox isoform expression by immunoblotting and to determine Nox localization in the cells, since specific antibodies cannot be obtained. Myc and EGFP epitope tags were placed either at the N-terminus or C-terminus of Nox4 and Nox5. These plasmids were expressed in HEK293 and Cos cells and compared with untagged Nox4 and Nox5 for ROS generation. We noticed that Nox4 is constitutively active in untagged and N-terminal tagged form, while C-terminal tagged Nox4 and all versions of Nox5 did not release ROS into the medium (as measured with the HVA assay). Intracellular ROS production was measured with the nitroblue tetrazolium assay and showed that transfected Nox5 did not produce ROS under these experimental conditions. We transfected also the Nox5 long form into HEK293 cells and observed basal as well as ionomycin-stimulated ROS generation. We decided to pursue the Nox4 isoform for our studies until we confirm which Nox5 isoform is clearly present in cancer cells. We suspect that small alterations in the Nox sequence may allow constitutive activity. To evaluate how the presence of $p22^{phox}$ may affect Nox-dependent ROS generation, we transfected Nox4 into two different Cos cell lines. One line has very low basal levels of $p22^{phox}$, while the other is stably transfected with $p22^{phox}$. The higher level of $p22^{phox}$ protein increased ROS production by Nox4 about 30%, presumably through stabilizing Nox4 expression or by altering Nox4 localization. We will be able to assess the $p22^{phox}$ effect on Nox4 in more detail by using the Nox4 epitope tagged protein for our studies. Nox4 contains a putative signal peptide at the N-terminus, which may be cleaved off during processing. Experiments are currently underway to determine if cleavage occurs. The results of these experiments will have implications on potential changes of our design regarding the location of the epitope tag in the Nox4 sequence.

Objective 1: Evaluation of ROS as second messengers in cancer cell metastasis

We were not able to correlate the migratory behavior of different, ROS-producing breast cancer cell lines to the presence of Nox isoforms (see also Objective 3). We encountered also problems to assess migration and invasion of breast cancer cells with the *in vitro* assay systems commonly used. ROS generation in cancer cells was serum-dependent (Figure 4), which posed a problem for long-term migration, invasion and attachment studies which are usually done without serum. To utilize a cleaner system, we established several Nox4- or vector control-expressing HEK293 cell lines by hygromycin selection (Figure 5). As shown all seven Nox4-expressing clones produced ROS constitutively, while the control cells showed no production. Nox4-dependent ROS generation is also serum-dependent as observed with ROS production in cancer cells. Interestingly, we detected an increase in ROS generation by Nox4-expressing cells

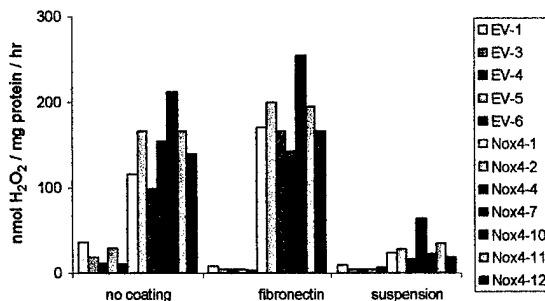


Fig. 5: ROS generation of HEK 293 cell lines stably expressing vector only (EV) or Nox4 on different matrices.

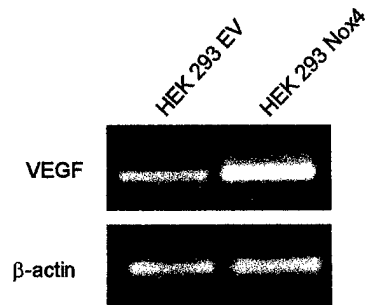


Fig. 6: RT-PCR to detect VEGF message in HEK 293 vector (EV) or Nox4 expressing cells.

when plated on fibronectin instead of plastic, and an almost complete down-regulation of ROS generation upon detachment. Detachment was achieved by plating cells on matrices which do not allow any cell attachment. This seems to be an important regulatory mechanism for ROS production by Nox4 and our further studies will pursue this line of investigation instead of evaluating ROS-dependent activation of transcription factors. Important prerequisites of metastasis are not only cell movement or degradation of the extracellular matrix, but also stimulation of angiogenesis. Injection of NIH 3T3 fibroblasts stably expressing Nox1 into mice leads to tumor formation, which is characterized by highly vascularized tissue (6). We tested HEK293 cells expressing the control vector or Nox4 for up-regulation of VEGF. Figure 6 shows that while the control β -actin message levels were equal, VEGF message was clearly up-regulated in Nox4 expressing cells. We have not yet tested the release of VEGF into the culture medium, but would predict an increase.

KEY RESEARCH ACCOMPLISHMENTS:

- RNA isolation and RT-PCR from breast epithelial, breast cancer cells and control cells to detect *nox* genes and NADPH oxidase components.
- Expression of various forms of Nox4 and Nox5 in epithelial cells to assess Nox isoform regulation and function.
- Analysis of stably transfected Nox4-expressing cell lines and control cell lines to evaluate parameters for cell adhesion, ROS generation and VEGF up-regulation.

REPORTABLE OUTCOMES:

Development of Nox4-expressing and control cell lines. We need to accomplish additional work on Nox regulation and function prior to publication or presentation at meetings. A poster will be presented at the Era of Hope meeting in Florida, September 25-28, 2002. The Principal Investigator is co-organizer for a Cold Spring Harbor Conference on Nox proteins in late fall 2002.

CONCLUSIONS:

Several Nox family members have been detected in cancer cells, but also in immortalized breast epithelial cells. Their functions and regulation are still unknown, but are implicated in promoting cell growth, tumor formation in mice and possibly anti-bacterial defense (8). Characterization of Nox protein regulation and subsequent functions in less complex systems will be necessary to shed light on their altered regulation in cancer cells. ROS have been implicated in tumor metastasis and angiogenesis in mice and the deregulated or altered state of Nox proteins in cancer cells may mediate these effects.

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