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13. ABSTRACT (Maximum 200 Words)

This project studies a novel apoptosis pathway that is induced by the death domain of the adaptor protein FADD (FADD-DD). This pathway can induce apoptosis only in normal epithelial cells from the breast (or prostate) and its inactivation may represent an early defect that arises in breast cancer. SV40 T antigen can selectively block this apoptosis pathway. The overall goal of this project is to investigate and further understand why breast cancer cells are resistant to this apoptosis pathway. As outlined in the previous progress report, we achieved all the goals for aim 1 and made new observations that allow alternate approaches to address the project. Importantly we showed that mouse breast epithelial cells display the same apoptosis response to FADD-DD. The reviewer of our previous progress report suggested that we study this response in the absence of SV40T antigen since this would be more applicable to actual breast cancer. We therefore continued with our studies as outlined in the statement of work and expanded our studies in mouse cells that allow us to avoid the use of SV40 T antigen. Our data show that inactivation of the p53, Rb, INK4a/ARF pathways do not explain resistance to this apoptosis.

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Introduction.

Evasion of apoptosis is a hallmark of cancer (Hanahan and Weinberg, 2000). Consequently, it is sometimes thought that cancer cells are generally resistant to apoptosis while normal cells are sensitive. In fact cancer cells are actually closer to their apoptotic threshold than their normal counterparts and therefore often undergo apoptosis more easily in response to diverse apoptotic stimuli (Evan and Vousden, 2001). This apoptosis sensitization occurs because growth promoting oncogenic events such as Myc expression raise the levels of caspases and other apoptotic proteins or make it easier to activate these molecules and thus reduce the threshold at which apoptosis is activated. However, it is not clear if this is the only apoptotic barrier that cancer cells must overcome as they become transformed. Are there also specific apoptosis pathways that inhibit cancer development and are active in normal cells and specifically inactivated during tumor development? We hypothesized that such a pathway would have the unusual characteristic of working in normal cells but not in cancer cells. Furthermore unlike most apoptotic stimuli, which usually work better in cancer cells than their normal counterparts because they are closer to their apoptotic threshold, signaling proteins and physiological stimuli that activate this kind of pathway should induce specific apoptosis responses in normal cells that should be selectively inhibited in cancer cells without affecting responsiveness to other apoptotic stimuli. The apoptosis pathway we study that is induced by FADD-DD has these characteristics because it works in normal epithelial cells but does not work in immortalized epithelial cells. Resistance to this pathway can be achieved by expression of SV40 Large T antigen. The established mechanism of FADD-induced apoptosis involves activation of caspase-8, however we previously showed that our pathway does not involve caspase-8 indicating that this is a novel mechanism of FADD-dependent apoptosis. Furthermore, as outlined in our previous annual report (April 2003), the resistance to this apoptosis pathway that arises in immortal cells is specific in the sense that it does not affect other apoptotic pathways including those induced by a FADD molecule that can activate caspase-8. The goals of this project were: 1) To determine whether T/t antigen alone can confer resistance and 2) To determine why T/t antigen causes resistance. Together, these experiments were intended to determine why breast cancer cells are resistant to this apoptosis pathway

Body.

Achievements towards goals in approved statement of work.

As described in the April 2003 report, we achieved our goals for aim 1 and showed that Large T alone can confer resistance to FADD-DD, we also achieved the first part of our goals (task 2a) for aim 2. Tasks 2b and 2c, were intended to address the mechanism if small t antigen was involved in the resistance mechanism. However our previous experiments showed that small t plays no role in this response and we therefore answered this question. The remaining goals were to determine if known activities of large T antigen (p53 inactivation (task 2d, to be completed in months 15-24), Rb inactivation and CBP inactivation, (task 2e and 2f) to be completed in months 24-36) were responsible for the resistance mechanism. Of these three mechanisms, we thought that the most likely is p53 inactivation because p53 is known to work by inducing apoptosis.

Our goals as described in the approved SOW for this funding period were to achieve tasks 2a-d. As outlined above, we had already achieved our goals for tasks 2a-c. We have therefore focused on task 2d (to determine if p53 signaling is required). We have also been able to achieve task 2e (to determine if Rb signaling is required), which was to be addressed in year 3. This rapid progress has been achieved because in response to the previous review, we found a way to avoid reliance on the SV40T antigen system.

Suggestions of the reviewer of the previous progress report.

The reviewer of the previous progress report stated "...this reviewer understands that the SV40 T/t antigen context is enshrined in the SOW, and the PI is faithfully carrying out his obligations under the contract, the work would be more meaningful if the SV40 system could be viewed in the context of non-SV40 transformed breast cells.....". We agree with the reviewer and as requested by Ms. Pawlus in the review of the previous report (25 August 2003), have taken the reviewers concerns into account. We have been able to make progress in this regard because as first reported in April 2003, we discovered that primary mouse breast epithelial cells (MMEC) show the same response as human cells to FADD-DD. That is, primary cells undergo apoptosis in response to FADD-DD but immortalized cells do not. In addition, this effect is epithelial-specific (see Fig 1).

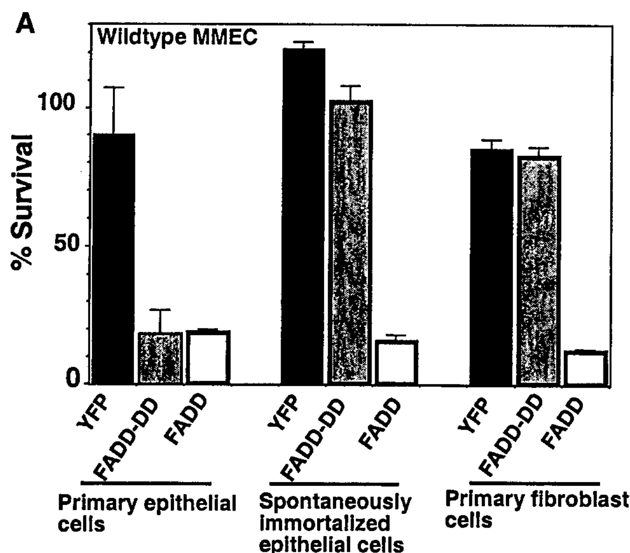

Figure 1. Primary mouse breast epithelial cells undergo apoptosis in response to FADD-DD. Primary or spontaneously immortalized MMEC were cultured and injected with expression vectors encoding YFP, or YFP-tagged FADD-DD or a FADD molecule that can activate caspase-8, which serves as a control for specificity. In addition primary breast fibroblasts were tested. FADD-DD, which can only activate the novel pathway and cannot activate caspase-8, induced apoptosis only in the primary epithelial cells. IN contrast, the FADD molecule that can activate caspase-8 induced apoptosis in all cell types. These data show that the FADD-DD pathway works in mouse cells as it does in human cells and demonstrate that spontaneously immortalized cells become selectively resistant to the FADD-DD apoptosis pathway even in the absence of SV40T antigen. Thus, we can study the mechanism of selective resistance to the FADD-DD pathway without the complications associated with reliance on the SV40 system.

By using mouse cells, we have been able to achieve tasks 2d and 2e with more scientific rigor than would be possible with our initial plan, which relied on other viral proteins

such as HPV E6 and E7, extend our studies to address other signaling pathways (particularly the role of the INK4a/ARF tumor suppressor locus, which regulates both the p53 and Rb pathways) that were not in the original SOW. Moreover, as suggested by the reviewer, we have been able to avoid reliance on the SV40 system.

Task 2c. ....determine whether p53 signaling is required usingE6 or alternate methods to inhibit p53 (months 15-24).

To address this question, we obtained breast tissue from p53 knockout mice, cultured primary epithelial cells and tested if they underwent apoptosis in response to FADD-DD. If inactivation of p53 signaling by T antigen was responsible for the resistance observed previously, we should find that primary cultures of p53 knockout cells would be resistant to FADD-DD. However, if these cells are resistant to other apoptotic stimuli, such an effect would not be specific. We therefore also tested if the cells are resistant to a FADD molecule that can activate caspase-8. By using knockout cells rather than E6, we avoid the complication associated with using the viral protein, which may not completely inactivate p53 signaling and is known to have other effects that could complicate the interpretation of our results. Figure 2 shows that primary low passage MMEC (passage number 2-10) from p53 knockout animals undergo efficient apoptosis in response to both FADD-DD and the FADD molecule that can activate caspase-8. These data indicate that p53 signaling is not required for FADD-DD-induced apoptosis and definitively achieve our goals in task 2d.

As expected, p53 loss promotes immortalization of the cells and upon continued culture, these cells do not undergo senescence or "crisis" as is found with MMEC and other cells from wildtype animals but instead continue to proliferate in culture. Interestingly, continued culture of these cells (beyond ~ passage 20) leads to them becoming resistant to the FADD-DD apoptosis pathway. Moreover as was found in our previous experiments with both human cells expressing T antigen (see April 2003 report) and mouse cells (Fig. 1), this resistance is selective for the FADD-DD pathway since there is no inhibition of apoptosis induced by a FADD molecule that can activate caspase-8. These data also suggest that selective resistance to the FADD-DD pathway confers a selective advantage to the cells even when they do not undergo "crisis". If this interpretation is correct, it further suggests that the apoptosis pathway that we are studying must be inactivated for efficient growth of breast cancer cells.

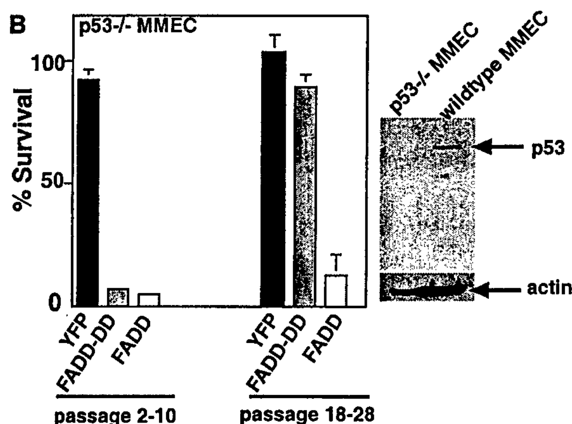

Figure 2. Inactivation of p53 does not confer resistance to the FADD-DD pathway. Primary MMEC were cultured from p53 knockout animals and tested for sensitivity to FADD-DD and FADD-induced apoptosis. Low passage cells were sensitive to both FADD-DD and FADD indicating that p53 signaling is not responsible for FADD-DD-induced apoptosis. Continued culture of these cells (~passage 18 and above) makes them resistant to FADD-DD-induced apoptosis but does not affect the response to a FADD molecule that can activate caspase-8. The western blot shows that the p53 knockout cells do not express any p53 confirming that the genotype of these cells is as expected.

Task 2d...to determine whether Rb signaling is required using E7 or alternate methods to inhibit Rb (months 24-30).

We took a similar approach to determine if Rb-deficient cells were resistant to FADD-DD. Because Rb knockout results in embryonic lethality (Jacks et al., 1992), we isolated MMEC from animals with homozygous floxed Rb genes. These cells were infected with an adenovirus that expresses Cre recombinase to knockout the Rb gene. Three days after infection there was no detectable Rb protein in the cells (Fig. 3 insert). FADD-DD injection into Rb-/- cells resulted in apoptosis induction that was equally efficient as that observed with the FADD molecule that can activate caspase-8. These data indicate that Rb signaling is not required for FADD-DD-induced apoptosis and address the goals outlined in task 2d.

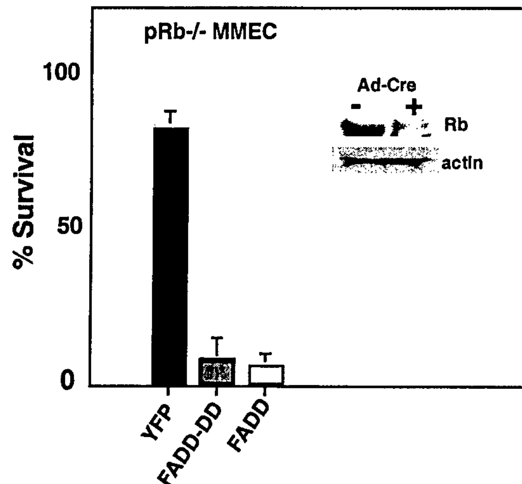

Figure 3. Inactivation of Rb does not alter responsiveness to FADD-DD-induced apoptosis. Primary MMEC were isolated from animals with "floxed" Rb genes and infected with an adenovirus expressing Cre recombinase this causes knockout of the Rb gene as indicated on the western blot. Responsiveness to FADD-DD-induced apoptosis was determined as before indicating that Rb-deficiency does not alter the apoptotic response.

Other achievements.

To extend these studies and further complete tasks 2d and 2e, we obtained MMEC from animals with a knockout of both the INK4a and ARF genes and the p53 target gene p21. These genes have been implicated in regulating cell cycle and apoptosis responses and the INK4a/ARF locus is specifically targeted during cell immortalization. The INK4a and ARF genes regulate the Rb and p53 pathways respectively thus the knockout of both genes (which are encoded by the same locus) simultaneously inactivates both the p53 and Rb pathways. Figure 4 shows that in both cases, the primary MMEC from these animals retained responsiveness to FADD-DD-induced apoptosis.

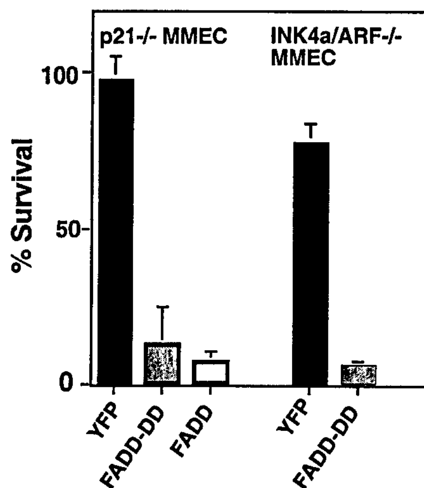

Figure 4. Inactivation of p21 or the INK4a/ARF genes does not confer resistance to FADD-DD-induced apoptosis. Primary MMEC were isolated from animals with knockouts as indicated and tested for FADD-DD-induced apoptosis as before. Both cell types respond like wildtype cells and are killed by FADD-DD.

Key Research Accomplishments (year 2).

- We achieved all our goals as outlined in the statement of work for years 1 and 2 and made progress towards our other goals by also completing task 2d, which was planned for the beginning of year 3.
- We confirmed that mouse breast epithelial cells respond to FADD-DD in the same way as human breast epithelial cells. That is, normal primary cells undergo apoptosis while immortalized cells do not. Importantly, this effect is highly specific since other apoptotic stimuli, including even a FADD molecule that can activate the caspase-8 pathway still induce apoptosis normally in these cells. This allows us to avoid complete reliance on the SV40 system, which was weakness in our original research plan.
- We showed that complete inactivation of p53 signaling (by genetic knockout of the p53 gene) does not prevent FADD-DD-induced apoptosis in primary breast epithelial cells.
- We showed that continued culture of p53 knockout cells leads to selective resistance to FADD-DD-induced apoptosis suggesting that subsequent loss of this pathway provides a selective advantage to cancer cells.
- We showed that complete inactivation of Rb signaling (by genetic knockout of the Rb gene) does not prevent FADD-DD-induced apoptosis in primary breast epithelial cells.
- We showed that complete inactivation of both the INK4a and ARF genes, which target both the Rb and p53 pathways does not prevent FADD-DD-induced apoptosis in primary breast epithelial cells. Inactivation of the p21 cell cycle regulator also has no effect.

Reportable outcomes.

None. This work is being prepared for publication but the manuscript has not been submitted at this time.

Conclusions.

We have continued to make progress in this project. We achieved all our objectives for aim 1 in year 1 and have now achieved all our objectives for year 2 and made inroads into the objectives for year 3. We were able to avoid the weakness that was identified by the reviewer of the previous annual report and avoid our previous reliance on the use of the SV40 system (or other viral systems) to examine how normal breast epithelial cells become resistant to this apoptosis pathway. By using genetic knockouts rather than T antigen or other viral proteins, to test the role of p53, Rb etc., we have been able to avoid the possibility that incomplete inactivation of the target or alternate targets of the viral proteins lead to misinterpretation of our data. Our data show that the FADD-DD pathway becomes selectively inactivated in immortal epithelial cells. This may represent a very early defect in that arises in the development of breast cancer. Furthermore because the known targets that regulate immortalization (TERT, addressed in year 1), p53, Rb, INK4a and ARF (addressed here) do not explain the immortalization-dependent resistance, these data imply that a previously unidentified target that is important in the immortalization process regulates sensitivity to FADD-DD.

What does this mean for breast cancer? This work addresses a fundamental question in breast cancer biology; what is the nature of the apoptotic defects that are required for cancer development? The fact that selective resistance to the FADD-DD apoptosis pathway arises at a very early stage in cancer development and that this is achieved through mechanisms that do not involve the known targets that are inactivated during cell immortalization suggests that this may represent a new signaling pathway that connects apoptosis regulation to continued tumor cell growth. The re-activation of such a pathway in cancer cells would be anticipated to selectively kill breast cancer cells. We therefore think that further understanding of how this pathway works as outlined in our current SOW and in other projects in our laboratory that address different aspects of this problem may identify new therapeutic targets that could be useful for future breast cancer treatments.

References.

Evan, G.I., and K.H. Vousden. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature*. 411:342-8.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Jacks, T., A. Fazeli, E.M. Schmitt, R.T. Bronson, M.A. Goodell, and R.A. Weinberg. 1992. Effects of an Rb mutation in the mouse. *Nature*. 359:295-299.