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13. ABSTRACT *(Maximum 200 words)*

This research distinguishes mechanisms through which activated Ras initiates rat mammary gland carcinogenesis. The initial experiment expressing H-Ras and K-Ras chimeric proteins in rat mammary gland (Aim 1A) supports the hypothesis of Aim 1; (the difference in rat mammary gland carcinogenesis results from differences in the last 20 amino acids of H-Ras and K-Ras). However, early results of the repeat experiment are beginning to weaken the hypothesis; it is still to soon to tell.

Aim 2A, expressing individual Ras effector loop mutants (ELM's), which target particular Ras effectors, substantially weakens the hypothesis of Aim 2; (No individual Ras effector will initiate transformation, rather multiple effectors must synergise). Each of the Ras ELM's (G37 activates RalGDS, E38 activates Raf, and C40 activates PI3K), individually causes tumors.

The results of Aim 1B, expression of Raf activated by fusion with Ras membrane localization signals (Raf-Caax), contrast surprisingly with results from Aim 2A. None of the forms of Raf tested resulted in a single tumor, yet E38-Ras, which activates Raf, is very tumorgenic. These results suggest Raf-Caax is defective for *in situ* tumor formation, or that E38 Ras is activating an additional effector pathway(s).

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FOREWORD

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Introduction:

This research distinguishes mechanisms through which activated Ras induces rat mammary gland carcinogenesis. Aim 1 focuses on differences between H-Ras and K-Ras, while Aim 2 focuses on different Ras effector pathways. In situ expression of activated H-Ras is 5-10 times more tumorgenic than K-Ras in rat mammary gland. Experiments expressing H-Ras and K-Ras chimera proteins in rat mammary glands are distinguishing critical differences between H-Ras and K-Ras proteins. Activated Ras proteins bind other signal transduction proteins, such as Raf, PI3K, and RalGDS. We don't know which Ras effector proteins are necessary and/or sufficient for initiation of mammary tumors. *In situ* expression experiments with Ras effector loop mutants (ELM's), and Raf-Ras fusion proteins (Raf-H-Caax, Raf-K-Caax), are distinguishing critical Ras effector proteins. These results have important implications for targeted treatment of different tumor types with novel chemotheraputic agents.

Body:

Aim 1 is to distinguish which H-Ras and K-Ras protein domains result in different potential to transform *in situ* mammary epithelial cells. The hypothesis of Aim 1 is; the different potential of H-Ras vs. K-Ras to initiate rat mammary gland carcinogenesis, results from differences in the last 20 amino acids of H-Ras and K-Ras. This hypothesis is being tested with expression of chimeric Ras proteins.

Aim 1A is comparing differences in rat mammary tumor formation resulting from expression of activated H-Ras and K-Ras with their carboxyl ends exchanged. The initial experiment supports the hypothesis of Aim 1; K-Ras with an H-Ras carboxyl end, resulted in many tumors (like H-Ras), and H-Ras with a K-Ras carboxyl end, resulted in few tumors (like K-Ras). However, early palpation results from the repeat experiment are beginning to weaken the hypothesis. If the early trend of the repeat experiment continues, further experiments may be necessary to determine significance.

Aim 1B was to compare differences in rat mammary tumor formation resulting from expression of Raf activated by fusion to the carboxyl end of H-Ras (Raf-H-Caax) vs. Raf activated by fusion to the carboxyl end of K-Ras (Raf-K-Caax). The results of Aim 1B don't reflect on the hypothesis of Aim 1, since neither form of Raf resulted in a single tumor. The results of Aim 1B strengthen the hypothesis of Aim 2: no individual Ras effector will initiate transformation, rather multiple effectors must synergise.

New antibodies and fractionation techniques that allow staining for individual Ras family members and fractionation of plasma membrane micro domains make the methods proposed for Aim 1C antiquated. These newer methods will be employed if follow-up Aim 1A experiments validate the hypotheses of Aim 1.

Aim 2 is to distinguish the pathway(s) critical to transformation of *in situ* mammary epithelial cells by activated H-Ras. As mentioned earlier, the results of Aim 1B strengthen the hypothesis of Aim 2: no individual Ras effector will initiate transformation, rather multiple effectors must synergise. Raf is thought to be the most tumorgenic of Ras effectors, so lack of tumor formation from expression of activated Raf, suggests Raf must synergise with an additional Ras effector(s). The results from Aim 1B contrast with those from Aim 2A.

Aim 2A was expressing individual Ras effector loop mutants (ELM's), which target particular Ras effectors (G37-Ras activates RalGDS, E38-Ras activates Raf, and C40-Ras activates PI3K). The results of Aim 2A experiments substantially weaken the hypothesis of Aim 2. Each of the Ras ELM's causes tumors individually. Tumors from expressing G37 or C40 Ras had a long latency (12-16 weeks), similar latency to tumors from expression of non-activated wt-H-Ras. E38-Ras, which activates Raf, is very tumorgenic with a short latency (3-5 weeks), as seen with activated H-Ras. This suggests Raf is the critical tumor generating effector of activated H-Ras. The coexpression experiments proposed for Aims 2B and 2C are no longer relevant due to the results of Aim 2A.

The contrasting results from Aim 1B and Aim 2A suggest Raf-Caax is defective for *in situ* tumor formation, or (and) E38-Ras is activating an additional effector pathway(s). RT-PCR of mRNA from Raf-H-Caax or Raf-K-Caax infused glands, shows some cells in the gland are still expressing the RNA months after infusion. Transformation of cultured cells by the Raf-Caax vectors, and immunostaining of infected cells has confirmed expression of the Raf proteins from the vectors. One possibility, the rats are having an immunoresponse to cells transformed by expressing the Raf-H-Caax protein. The Raf proteins expressed so far all carried an epitope tag for future identification. Raf expression vectors are being reconstructed without the tags to rule out an immunoresponse. In addition, Raf-H-Caax Infused glands are being sectioned and checked for infiltration by immune cells, and blood was collected from Raf-H-Caax infused animals to check for antibodies to the tag.

A great deal of current research involving Ras, is working to identify additional effectors: results from that work will do more than I can, to determine if E38-Ras is activating another effector(s) besides Raf. I will be able to check the tumors generated by

expression of different Ras-ELM's for activation of known pathways. Antibodies will be used to check for activation of appropriate pathways in Ras ELM tumors. Immunofluorescence for Erk, Jnk, and Raf, on sections from tumors generated by expression of the different Ras ELM's and activated H-Ras will help determine which pathways are active in each. Immunoprecipitations and westerns (with antibodies to Ras, Raf, PI3K, phospho-Erk and phospho-Jnk) are also being pursued to characterize which pathways are active and which proteins are associating in different Ras ELM tumors.

Key research accomplishments:

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-H-Ras-H-Caax, JR-H-Ras-K-Caax, JR-K-Ras-K-Caax, and JR-K-Ras-H-Caax retroviral vectors (domain swap vectors).

- Infused glands with domain swap vectors and palpated; there were 14 or 15 rats per group.
  --Suggests critical differences lie in carboxyl ends.

- Infused glands with domain swap vectors and have begun palpating; there were 12 rats per group.

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-Raf-H-Caax, JR-Raf-K-Caax, and JR-wt-Raf retroviral vectors (Raf-Caax vectors).

- Infused glands with Raf-Caax vectors and palpated; there were 12 rats per group.
  --Suggests Raf alone is not able to transform mammary epithelial cells *in situ*.

- RT-PCR on RNA from Raf-Caax infused glands.
  --Confirmed expression of Raf-Caax RNA's in infused mammary glands.

- Immunostaining of Raf-Caax infected cultured cells.
  --Confirmed expression of Raf-Caax proteins in infected cultured cells.

- Constructed, concentrated, titered, and tested for absence of helper virus in JR-V12-H-Ras, JR-G37-V12-H-Ras, JR-E38-V12-H-Ras, and JR-C40-V12-H-Ras retroviral vectors (Ras-ELM vectors).

- Infused glands with Ras-ELM vectors and palpated; there were 12 rats per group.
  --Suggest Ras can generate tumor formation by activating any individual effector.
  --Suggest Raf activated by Ras (without PI3K or RalGDS) transforms like activated Ras (fast), while PI3K or RalGDS activated by Ras transform like non-activated wt-H-Ras (slow).

- Infused glands with JR-V12-H-Ras or JR-E38-V12-H-Ras ELM vector and palpated, there were 12 rats per group.
  --Confirmed Raf activated by Ras is similar to transformation from activated Ras.

- Infused glands with Ras-ELM or Raf-Caax vectors and palpated; there were 12 rats per group.
  --Confirmed Raf activated by Ras (without PI3K or RalGDS) transforms like activated Ras, while Raf-Caax fails to transform.
  --Confirmed PI3K or RalGDS activated by Ras, results in much greater latency to tumor formation, than Raf activated by Ras, or activated Ras.

Reportable Outcomes:

McFarlin, D.R., Kennan, W.S. and Gould M.N., "Ras signaling through Raf is more tumorgenic in rat mammary gland than Ras signaling through PI3K or RalGDS."; Abstract #2475 in Proceedings of the American Association for Cancer Research, vol.40, p.374, 1999.

This was presented as a poster at the 90th annual meeting of the American Association for Cancer Research in Philadelphia, PA in April of '99.

A similar poster presentation was given at the "Genetics, Genomes, and Molecules" symposium on the UW-Madison campus in May of '99

Copy of Reportable Outcome:

Ras signaling through raf is more tumorgenic in rat mammary gland than ras signaling through PI3K or RalGDS. McFarlin, D.R., Kennan, W.S. and Gould, M.N. *McArdle Laboratories for Cancer Research, University of Wisconsin, Madison, WI 53792*

Ras proteins are guanine nucleotide binding proteins localized at the plasma membrane. In its active state, the effector loop of Ras binds many other signal transduction proteins including Raf, PI3K, and RalGDS. Ras with a valine substitution for glycine at amino acid 12 (V12-Ras) is continually active and oncogenic. Ductal infusion of rat mammary glands, with retroviral vectors conferring expression of V12-Ras, results in tumor formation from infected mammary epithelial cells (MEC). V12-Ras which also has a glutamic acid substitution in the effector loop at amino acid 38 (E38-V12-Ras) is not able to bind PI3K or RalGDS but is still continually active for binding Raf. Expression of E38-V12-Ras in rat mammary gland generates an equivalent number of tumors as expression of V12-Ras. C40-V12-Ras is not able to bind Raf or RalGDS but is still binds PI3K. Expression of C40-V12-Ras in rat mammary gland generates significantly fewer tumors than V12-Ras, or E38-V12-Ras. G37-V12-Ras is not able to bind Raf or PI3K but is still binds RalGDS. Expression of G37-V12-Ras in rat mammary gland generates a similar number of tumors as C40-V12-Ras and significantly fewer tumors than V12-Ras, or E38-V12-Ras. Equivalent tumor numbers resulting from expression of V12-Ras and E38-V12-Ras, substantiates Raf as a considerable contributor to transformation by Ras. Generation of significantly fewer tumors by expression of C40-V12-Ras and G37-V12-Ras suggests interaction of PI3K and RalGDS contribute less to MEC transformation by Ras than interaction with Raf.