Molecular Targets of Calcium and Vitamin D in Mouse Genetic Models of Intestinal Cancer

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We have identified intracellular targets that are affected by vitamin D in intestinal epithelial cells in two ways: analyzing profiles of gene expression in a matrix of dietary-genetic interactions in mouse models, and exploring a novel mechanism of transcriptional attenuation. Genetic models of intestinal cancer in the mouse models included inheritance of a mutation in the Apc gene (Apc1638N/H11001– mice) and the targeted inactivation in the mouse germ line of the Muc2 gene (Muc2–/– mice) that encodes the major gastrointestinal mucin. Each of these models is highly significant in terms of the etiology and mechanism of human intestinal cancer. Inheritance of an Apc mutation, with its subsequent reduction to homozygosity due to somatic alteration of the remaining wild-type allele, is the etiology of familial adenomatous polyposis. Moreover, targeting of Apc somatically, or of the Wnt pathway in which it has an important role, is associated with almost all colorectal cancers. Muc2 mutations have not yet been found in human colon tumors, but in the mouse Muc2 model, goblet cell differentiation is perturbed and the protective mucus layer in the small and large intestine is compromised. In this regard, goblet cells and the mucin they produce are often depleted in aberrant crypt foci (ACF), the earliest precursors to colon tumors. Thus, the mucosa of the Muc2–/– mouse may mimic focal changes that occur in development of human colon cancer (Velcich, unpublished observations). While the mechanism by which targeted inactivation of Muc2 in the mouse causes tumor formation throughout the intestinal tract is not entirely clear, it may involve chronic low-level inflammation in the mucosa and/or exposure of intestinal epithelial cells to genotoxic substances and cytokines. What is clear is that the mechanism of tumor development in Muc2–/– mice differs from that in Apc1/2 mice: there is no activation (e.g., nuclear accumulation) of β-catenin in the mucosal epithelial cells of the Muc2–/– mice or the tumors that form, and the introduction of an Apc mutation into the Muc2–/– mice greatly increases the number of tumors, indicating that the two mutations are at least complementary, and perhaps synergistic (Velcich and Yang, unpublished observations).

Mouse genetic models such as these have been fundamental for understanding the etiology and mechanisms of intestinal tumorigenesis. However, the vast majority of human colonic tumors are “sporadic,” indicating that there is no known genetic predisposition. Instead, a single tumor generally develops in about 25% to 50% of the population in the United States and other Western countries over six to seven decades of life—about two-thirds of the human life span. During this time, there are highly significant increases in the probability of individuals developing a single colonic tumor if they consume diets with characteristic amounts of a number of various macro- and micronutrients. To study the effects of these dietary factors on tumorigenesis, Newmark designed a number of mouse “Western-style diets” that mimic the levels of consumption, on the basis of nutrient density, of major risk factors in the human Western diet. We have studied the original Western diet (WD) (high fat, low calcium and vitamin D), as well as a more recent new Western diet (NWD). The NWD is similar to the WD, but it is also low in providing methyl-donor groups for intracellular functions (choline, methionine, folate, and fiber). It is important to note that the levels of these risk factors in the diets are not abnormally high (fat), nor low (calcium, vitamin D, choline, methionine, folate, fiber), but reflect levels that are associated epidemiologically in the general

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Western population with an elevated probability of tumor formation.

Our first experiments addressed the affects of the NWD on wild-type (WT) C57Bl6 mice. Mice fed a control AIN76A diet rarely developed small or large intestinal tumors, which is consistent with the low incidence of intestinal tumors in WT mice; whereas, when fed the NWD from weaning to 1.5 to 2 years of age, a single colon tumor developed in about 25% of the mice. Moreover, elevation of the lower levels of calcium and vitamin D in the NWD completely prevented this tumor formation, even though the other risk factors in the diet remained (e.g., higher fat). We suggest that this is a new model of sporadic colon cancer, in which tumors develop over two-thirds of the life span of the mouse in approximately 25% of the population exposed to the same dietary risk factor as in the human Western populations. Although the elevation of calcium and vitamin D in the diet was effective in preventing this tumor formation, the elevation of choline, methionine, folate, or fiber individually did not have significant nor consistent effects on tumor formation. This may reflect the fact that each contributes to the single carbon pool, so any one component is relatively ineffective in altering these levels. Using this model of diet-induced colon cancer, we are currently investigating three important public health issues: 1) whether the affects of calcium and vitamin D in inhibiting diet-induced tumors can be dissociated, 2) how long it takes mice on NWD to become committed to tumor formation, and 3) when, during the 2-year feeding, the tumors are sensitive to inhibition of tumor development and/or growth by elevation of calcium and vitamin D in the diet.

To dissect the mechanisms involved in tumor formation, we have generated an extensive matrix of databases of tumor formation and gene expression as a function of diet and genotype. For the C57Bl6 WT mice fed either the control AIN76A diet, NWD, or NWD+calcium/vitamin D, expression profiling for the flat intestinal mucosa was done at 6 months of age, well before the detectable tumors form at about 18 months of age. We also determined tumor incidence and multiplicity, as well as gene expression profiles of the flat intestinal mucosa, of the two genetic models of tumor initiation (Apc+/− or Muc2−/− mice) and how these were modulated by diet as discussed above. For this extensive matrix of data, each mouse genetic/dietary group encompassed at least 20 mice for analysis of tumor formation, and separate mice for which gene expression analysis (Affymetrix chips) was assayed utilizing RNA from isolated epithelial cells of the large (proximal+distal, combined) or the small (duodenum+jejenum, combined) intestine.

Unsupervised clustering of all the colonic and small intestinal gene expression data of the C57Bl6 mice (24 mice – 4 each for AIN76A, NWD, and NWD+Ca/vitD for the small and the large intestine) showed that the two tissues clustered completely separately and independently of diet. This is consistent with the profoundly different histology and functions of the large and small intestine. Unsupervised clustering of the colonic data from all mice demonstrated that the WT C57Bl6 mice fed the control AIN76A diet always clustered together, and that perturbation by either the Apc or Muc2 mutations or the different diets shifted this, but with some heterogeneity. Moreover, the C57Bl6 mice clustered much closer to the Apc mice than to the Muc2 mice. Pursuing this, we calculated the means of the large intestine data for each gene for all four mice in each of the nine genetic/dietary groups (C57Bl6, Apc+/− or Muc2−/− fed either AIN76A, NWD, or NWD+calcium and vitamin D) and then clustered on the means. Clustering was primarily by genotype, even though, as discussed above, diet was a major determinant of tumor formation (and see below). The C57Bl6 and Apc+/− groups were on the same major branch of the cluster, and thus much more similar to each other, than was Muc2−/−, which appeared on a separate branch.

This clustering was reflected in the overlaps of gene expression changes among the three different etiologies of “initiation”: the dietary initiation (NWD compared to AIN76A in C57Bl6 mice), or either of the two genetic models of initiation (Apc+/− or Muc2−/− mice compared with C57Bl6 fed AIN76A). In summary, there were over 5000 sequences that were altered in expression by at least one of the genetic or dietary initiations. Expression changes associated with the NWD overlapped 75% with those induced in the flat mucosa by Apc, but only 57% with those induced by Muc2 inactivation. Interestingly, of 66 sequences that changed in opposite directions for Apc and Muc2, 35 did not change with the NWD, but 26 changed with the NWD as they did for Apc, while only five changed as for Muc2. Thus, the NWD drives cells along pathways more similarly to Apc initiation than to Muc2 initiation. This is also illustrated by clustering of data for each of the mice on the basis of only these “initiator” genes. Again, C57Bl6 mice fed the AIN76A diet all clustered together. With one outlier, Apc-initiated mice were bracketed on the same branch by NWD-initiated mice, with Muc2-initiated mice on a separate branch, thus confirming the greater similarity between Apc and NWD “initiation.”

Since the introduction of an Apc mutation into Muc2 mice greatly accentuates tumor formation, these array data predict that the NWD would likewise stimulate tumor formation when fed to the Muc2 mice. Moreover, since the NWD is more similar in terms of gene expression profile to Apc initiation, we predicted that the NWD...
would more readily complement Muc2 than it does Apc; therefore, the effect of the NWD diet on colon tumor formation when fed to Apc<sup>+/−</sup> mice would be less than the effect when fed to Muc2<sup>−/−</sup> mice. We have demonstrated that these predictions are correct. We have also determined that these observations made for the colon are true of the small intestine—that is, the NWD drives small intestinal epithelial cells along similar (but not identical) pathways as does Apc mutation, and therefore that the NWD has more pronounced affects on small intestinal tumorigenesis in the Muc2 mice, with which it is complementary, than on tumorigenesis in the Apc mice.

Finally, since dietary-initiated tumor formation in the C57Bl6 mice is reversed by raising the calcium and vitamin D levels in the NWD, we have identified sequences for which expression closely tracks calcium/vitamin D levels in the diet and hence probability of tumor formation. These sequences are greatly enriched in genes involved in intermediary and energy metabolism, which is consistent with the higher fat content of the NWD. Interestingly, a number of genes associated with the tricarboxylic acid cycle are down-regulated in association with feeding the NWD. Among these are pyruvate dehydrogenase and 3-ketoacyl-CoA thiolase B, genes that encode the enzymes that catalyze generation of acetyl CoA from carbohydrates and lipids, respectively. Importantly, the expression of these genes is elevated when calcium and vitamin D levels in the NWD are elevated. Thus, the NWD may increase the relative level of glycolytic metabolism in the intestinal mucosa as a concomitant of elevated risk for tumor formation, which is then restored to more normal levels when calcium and vitamin D levels are elevated. This is under investigation.

The second approach we have taken to identifying genes that respond to vitamin D involves significant expansion of our previous work on attenuation of c-myc gene transcription by two physiological inducers of colon cell maturation: the short-chain fatty acid butyrate and vitamin D. Using a novel method of imaging of active transcription sites in the interphase nucleus, we had demonstrated that both of these agents recruit a pause, or block, to transcriptional elongation of the c-myc gene downstream of its initiation. This attenuation contributes significantly to the down-regulation of c-myc steady state levels that have been well-documented as a driver of cell cycle arrest and maturation. We have now repeated similar experiments for the cyclin D1 gene with similar results: in inducing cell cycle arrest and maturation of colon carcinoma cells, both butyrate and vitamin D caused an attenuation of transcriptional elongation of cyclin D1, contributing to the decline in cyclin D1 steady-state levels (Maier and Augenlicht, unpublished data).

To expand this work, we designed and fabricated a new chip that interrogates the 5′ and 3′ end of each of the more than 17,000 sequences in the RefSeq database (i.e., sequences whose genomic/transcriptional structure are understood). This identified several hundred sequences for which the 3′ end was down-regulated following butyrate treatment, but for which the 5′ end was up-regulated. Fewer sequences, comprising a non-overlapping, distinct data set, were also identified with this pattern of 5′ and 3′ expression in response to vitamin D3. A second generation chip has been designed that “tiles through” these sequences, as well as a set of control sequences, at a resolution of 5 to 20 nucleotides. This will permit us to: 1) confirm which genes exhibit transcriptional pausing in response to butyrate or vitamin D3 (or other nutrients); 2) distinguish these from genes undergoing differential splicing; 3) determine the site of transcriptional attenuation in the gene; and 4) dissect the cis and transacting factors that are important to the mechanism of attenuation.

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