Studies Regarding Intestinal Absorption and Action of Insulin-Like Growth Factors in the Newborn

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After all the discussions we had yesterday and this morning about whether IGF is absorbed from the milk, I feel like the proverbial deer in the headlights here. It reminds me of one of my mentors, Giacomo Meschia at the University of Colorado. During my fellowship we were pouring over some data when he remarked: "I think I have discovered the truth, but I don't understand it." And I think that once we are able to take a better look at all of the factors involved in IGF and milk, development issues in the newborn, gut closure, dosage and timing, we'll probably be able to reconcile all of this information. I suggest that all the disparities that we see are probably secrets yet to be unlocked.

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- Coworkers:
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  - Univ Utah: R. Lute, N MacLennan
  - Univ Wisconsin Madison: P Kling
  - UC Davis: G. Kelleher, S Kelleher

- Pharmaceutical colleagues:яв

The work I will be telling you about occurred over a period of 15 years. It was supported by a number of different institutions, including the National Institute of Health. My coworkers include Bohuslav Dvorak and the late Otakar Koldovsky at the University of Arizona and my collaborators include Rob Lane at the University of Utah, and Bo Lonnerdal and Shannon Kelleher at UC Davis.

Prematurity Incidence

- Roughly 4M live births in U.S. per year
- Rate of Low Birth Weight = 7.9% ~ 350,000/yr
- Major problems
  - Respiratory
  - Nutritional
  - Anemia
  - Sepsis

Dr. Han and I are both neonatologists and we worry a great deal about premature babies. There are roughly 4 million live births in the United States every year, and the rate of low birth weight is somewhere around 7 or 8 percent. That's a lot of babies in the United States alone. Over the last 30 years we have made great progress dealing with respiratory problems in these babies, but we still have enormous difficulty with nutrition. We have difficulty getting them to grow, particularly in the first weeks after birth.

Premature Babies (<1500 gm) grow poorly postnatally due to poor nutritional intake

(Hack, et al PEDIATRICS Vol. 112 No. 1 July 2003, pp. e30-e38)

This slide shows some work by Maureen Hack that was in the literature a couple of years ago. It shows that from birth on, these very low birth weight babies are quite depressed in terms of their weight and height and it is weeks before they can achieve catch up growth and there is much variability. Some of them never do achieve catch up growth or achieve their potential.
Background

- Premature babies often exhibit poor growth in first weeks postnatally due to immature intestinal function.
- Mammalian breast milk known to contain at least 28 growth promoting substances, including Insulin-Like Growth Factors (IGF’s).
- Infant formulae are devoid of these, yet breast milk not available to many neonates on long term basis.
- Is it feasible to engineer artificial formula to make it more like breast milk?
  - Calcium, Phosphorus, glucose polymers, MCT’s; fat soluble vitamins and nucleotides to improve nutrient uptake.
  - Probiotics to guard against infection, under clinical trial investigation.
  - Biosynthetic calcium, phosphorus, glucose polymers, artificial formula to make it more like breast milk.

Milk and Insulin-Like Growth Factors

Hypotheses

To prove a milk borne substance has significant biological activity, substance in question should:
- remain “intact” in gastrointestinal tract (GIT)
- have biological effect(s) on GIT and/or at more distant sites

As Dr. Baumrucker pointed out, to prove that a milk borne substance has biological activity, you want to know a couple of things. You want to know whether it stays intact in the gut. And if it does stay intact, does it have a biological effect, either in the intestine or in more distant sites.

These low birth weight babies exhibit poor growth in the first weeks due to immature intestinal function. We now know that mammalian breast milk, as Dr. Baumrucker has shown, contains at least 28 different biologically active substances and that includes insulin-like growth factors (IGF) 1 and 2. We know that infant formula is devoid of most of these, particularly IGF-1. And in the United States where breast milk banking is not very common, breast milk for long periods of time is not available to many of our babies. So we wondered whether it was feasible, if you will, to engineer artificial milk to make it more like breast milk. A number of things have been done over the last ten years, including the addition of more calcium, phosphorus, glucose polymers, medium chain triglycerides (MCTs), fat-soluble vitamins, and nucleotides to improve nutrient uptake and growth. Probiotics that will guard against infection, particularly in these low birth weight babies are now under clinical trial investigation. Since growth factors are in milk but not in formula, we wondered whether some biosynthetic growth factors should perhaps be added. So much of this work was designed to try to answer this question.

Milk and Insulin-Like Growth Factors

As a number of us have said, IGF and their binding proteins (IGFBP) are present in the milk of many species. Colostrum has the highest concentrations. We know that IGF in vitro simulates mitosis in a number of cell types and does have some insulin-like metabolic effects. Injection and knock out studies, such as those by Dr. Han, suggest that it is essential for perinatal growth. And, now, a human mutation in the IGF-I gene has been identified. IGF-I is modulated by specific binding proteins. The IGF-I type 1 receptor is synthesized by many cell types, including in the gut, particularly in the jejunum and in the crypts. Yet very little intestinal IGF synthesis exists, which raises several interesting questions. Why are the receptors there? Is milk borne IGF used at that level? Is milk borne IGF-I absorbed?

??Breast milk fed neonates have higher serum IGF-I levels than those receiving formula

As Dr. Baumrucker pointed out, to prove that a milk borne substance has biological activity, you want to know a couple of things. You want to know whether it stays intact in the gut. And if it does stay intact, does it have a biological effect, either in the intestine or in more distant sites.

The seminal question from yesterday evening is, do breast-fed babies have higher serum IGF-1 levels than those who receive formula? Well, there is some animal data to suggest that is true, Doug Burrin in piglets, Craig Baumrucker in calves and our data that I will show you in the neonatal rat. Only very limited data exists for humans. Again, it's very controversial. But in this one study by Diaz-Gomez, they were only looking at premature babies under about 1,500 grams who were either formula or breast-fed. And the babies who were breast-fed had higher levels of IGF. The question is, if the levels are higher, is this due to endogenously produced IGF-1, or is it milk derived? And we don't really know the answer.

I show here some work that we did back in the late ’90s, in what has been called the “pup in the cup” model. We fed neonatal rats a rat milk substitute (RMS) that either had 500 ng/ml of

??Breast milk fed neonates have higher serum IGF-I levels than those receiving formula

- Piglets: Burrin et al 1995
- Calves: Baumrucker, 1994
- Rats: Philippus, et al 1997
- Humans: Diaz-Gomez et al 1998
- Prematures
  - ?? Term babies controary

The question is, if the levels are higher, is this due to endogenously produced IGF-1, or is it milk derived? And we don't really know the answer.
recombinant human IGF-I (rhIGF-I), a somewhat higher than physiologic dose, or RMS with no rhIGF-1 and we compared them to dam fed animals after three days of infusion. The dotted line represents the normal IGF-1 concentration of dam fed rats in our laboratories. The animals that received no rhIGF-1 supplement had IGF-1 levels that were about a third of the animals that received the supplement and the IGF-1 concentrations in these supplemented animals more closely approximated the dam fed control animals. So I will show you several studies that were done, either by acute injection or ingestion and then compare them to some of the chronic studies that we have done.

Milk and Insulin-Like Growth Factor-I
Acute and Chronic studies

**Suckling rat model**

- Acute injection of Biosynthetic IGF-I 1985-6 Stockholm

I want to point out this study, which first got me involved in IGF work. I was fortunate to be able to do a sabbatical in Stockholm with one of three groups in the world in 1985 that were looking at what were then called somatomedins. Working with Kerstin Hall and Vicki Sara, we were able to use the first biosynthetic available IGF-1 preparation and inject it into neonatal rats over a period of ten days. As you can see in the picture, these rats grew about 15 percent bigger than the animals we thought were growing optimally, and their liver and brain weights were increased. And the fascinating thing for me in particular was that their eyes opened two days earlier than the normal animals that were not injected.

Milk and Insulin-Like Growth Factor-I
Acute and Chronic studies

- Suckling rat model
  - Acute ingestion studies
  - Chronic feeding studies (“Pup in the cup”)

I will now show you a few ingestion studies that we have done. This is a photograph of the pup in the cup model. There is a refrigerated series of mechanized pumps that are all computerized, attached by long PBC tubing to baby rats that have been gastrostomized and put in these little floating cups in a water bath at about 34 degrees to maintain them. These are, as you might imagine, somewhat difficult experiments to do. Hopefully, they will provide us with a bit of insight as to what might be going on, at least in the neonatal rat.

### Milk and Insulin-Like Growth Factor-I
*Results 1990-2003*

- In comparison to suckling rats fed a Rat Milk Substitute (RMS), animals fed IGF-1 supplemented milk had:
  - Distal/Systemic effects:
    - Enhanced weight, body, liver, brain (by 5%+)
    - Normalized serum IGF-1 and IGF-BP-2 levels
    - Normalized serum glucose concentrations
    - Enterohepatic circulation of IGF-1

In comparison to the suckling rats fed RMS, animals fed IGF-1 supplemented RMS had several systemic effects including about 5% enhanced body weight and normal IGF-1 and IGF-BP-2 levels. BP-2 at this age in rats seems to be the predominant serum binding protein. And if I have time, I’ll talk about the glucose levels in these animals and whether there is what we call an enterohepatic circulation of IGF-1.

### Milk and Insulin-Like Growth Factor-I
*Results 1990-2003*

- In comparison to suckling rats fed a Rat Milk Substitute (RMS), animals fed IGF-1 supplemented milk had:
  - Local intestinal effects:
    - Biologically intact 30 min post ingestion
    - Increased gut growth
    - Stimulation of enterocyte migration from crypt
    - Intestinal metabolic “switch” from glycolysis to glucose transport

There were also some local intestinal effects in comparison to the suckling rats fed RMS. We found that IGF was present and in biologically active form at least 30 minutes post ingestion. We also found that gut growth was accelerated, at least more like the normal dam fed animals in comparison to the animals that received RMS. There was stimulation of enterocyte migration. The enterocytes develop in the crypts of Lieberkühn and then literally migrate up the villi and then are lost. And the animals that had RMS had clearly quite delayed enterocyte migration. And when we added the IGF-1, those animals had enterocyte migration rates that were similar to dam fed animals. And if there is time at the end, I will talk about what we found, which is an intestinal metabolic switch that seems to be dependent upon IGF-1 in the milk.
The reason we started looking at this enterohepatic circulation really relates to a finding of Wuyi Kong who was a postdoctoral researcher in our laboratory back in the late 1990s. She decided to look at bile in these samples based on a finding that bile IGF-1 was present in adult humans. So she conducted a series of cross-sectional studies of a number of animals of different ages that involved cannulating the bile ducts of even baby rats, which you can imagine is quite difficult. And she found that the bile IGF-1 was highest in the newborn and by adulthood fell to about a third of those levels. Knowing the bile flow rate, we calculated that about two-tenths of a microgram was presented to the gut in the newborn per day and about 1.6 micrograms in the adult rat. Adjusted for body weight, both newborn and adult were delivering about 10 ng per gram of body weight. When we added the amount of IGF-1 that was in the milk fed to the baby rats, we found that the total amount of milk plus bile derived IGF-1 that was being presented to the intestines in the suckling rat was equivalent to about half a microgram per day, which is about 25 to 30 ng per gram of body weight. So the total is quite a large amount, about two and a half times greater than bile IGF-1. The question is, what is the derivation of bile IGF-1. In a nutshell, we think it is probably not only of hepatic origin, but probably portal and maybe peripheral as well. The reason we say that is we did some acute injection studies of radioactive recombinant human IGF using I-125 labeled IGF. When Wuyi injected this into the peripheral circulation, she recovered a small amount, about 2 percent of the dose in bile. And when we checked this to see whether it was receptor active using a crude membrane preparation that had rat liver type 1 IGF receptor on it, we found that about 30 percent of that radioactivity actually stuck to the crude membrane receptor.

This is the study we did more recently to take a look at what might be happening if IGF-1 is actually absorbed and this is the study that was quoted, particularly in the cancer literature. Of course, if IGF-1 is absorbed, it doesn't really go into the peripheral circulation first, it goes into the portal circulation from the splanchic bed. So what we did in this acute study was to give suckling rats that same I-125 labeled rhIGF-1 by stomach tube. We then checked portal and peripheral blood at the same time in a cross-section of animals at either 5, 10, 15 or 20 minutes. In order to identify whether the radioactivity was IGF-1 or just counts, we did a couple of things. As we heard from some of the earlier presentations, extracting IGF-1 from milk is quite laborious. One has to separate the IGF from its binding proteins first, and that's what you see over on the left in this slide. When we put the blood of these animals over the Sephadex G50 column with acid, which separates IGF from its binding protein, we got this really significant peak of radioactivity that migrates in the position of what we will call native IGF-1. We did the same thing with high performance liquid chromatography and got the same peak in the same place where IGF-1 would elute. And then we took that peak and put it in a receptor assay. If we took the hormone that we were going to inject and put it in the assay, we had a binding to the receptor of about 30 percent, and when we added a large amount of cold unlabelled IGF, it displaced the activity of this radioactive hormone quite a bit, suggesting that it is biologically intact. If you look at the material that we extracted from the portal blood of these animals, we get a little bit lesser binding, about 20 percent, but, again, it is inhibited quite a bit by an amount of cold, unlabelled rhIGF-1. This suggests, but certainly doesn’t prove that it is biologically active. These are difficult studies to do with such a limited amount of sample.
Intestinal uptake of IGF-1 by Suckling Rats-Portal Circulation

- We hypothesize that:
  - If fed acutely, milk borne IGF-1 is absorbed into the portal circulation in a dose dependent manner and delivered to liver
  - If fed chronically, milk borne IGF-1 also enters the peripheral circulation
  - Some fractions of both are then filtered and delivered to bile, along with IGF-I of hepatic origin
  - Bile derived IGF-I is then presented to the small intestine in biologically relevant amounts, along with "new" milk borne IGF-I.

We hypothesized that if fed acutely, milk borne IGF-1 is absorbed into the portal circulation in a dose dependent manner in these animals and then gets delivered to the liver. What we don't know is if fed chronically would milk borne IGF-1 enter the peripheral circulation as well. If so, we think some fractions of both sources are then filtered by the liver and delivered into bile, along with IGF-1 that is of hepatic origin, and then bile derived IGF-1 is presented to the small gut in biologically relevant amounts, in addition to "new" milk derived IGF-1.

Milk Borne IGF and Intestinal Metabolism


- Initial finding: suckling rats fed RMS for 4 days have lower blood glucose levels than dam fed controls (180 ± 21 vs 105 ± 13 mg/dl)
- Addition of physiological amounts of IGF-1(50-100 ng/ml) to RMS returns blood glucose levels to near normal (153 ± 10 mg/dl)
- IGF-1 supplemented feedings brought intestinal glucose transporter (SGLT-1 and GLUT-2) mRNA and protein levels back to control levels found in dam fed pups, suggesting that they play a role in intestinal glucose uptake.

I also want to show you some initial findings by Rob Lane, at the University of Utah. While we were doing these studies we happened to have a blood glucose analyzer that was sitting up on the shelf unused. So we took it down and tested it and found that it could accurately analyze samples that were only 10 to 20 microliters. So we fed them this rat milk substitute with a much lower dose of IGF-1 this time, only about 50 ng/ml, which is pretty close to the amount in colostrum. To our surprise, we found that the dam fed animals had blood sugar levels that were about 180 milligrams per deciliter, but the animals that were fed RMS were down to about 100 mg/dl. The animals that received the IGF-1 at 50 ng/ml had blood sugar levels that were near normal at 150 mg/dl. We wondered why that was the case and Dr. Lane was kind enough to analyze some of the glucose transporters for us. He found that the IGF supplementation brought intestinal levels of the sodium dependent glucose transporter (SGLT1) and the major intestinal glucose transporter, GLUT2, back to the control levels in dam fed animals and that RMS seemed to suppress these. We followed this up with some work that Dvorak did at the University of Arizona and we found that the protein levels followed the messenger RNA levels quite closely.

Milk Borne IGF and Intestinal GLUT 2


This slide shows the GLUT2 expression in the villi and the red staining with confocal microscopy is the GLUT2 in these epithelial cells. Panel A shows the dam fed, B, the RMS, C, the RMS plus IGF-I and D, the RMS plus IGF-II. We actually did some work with IGF-II as well. As you can see the levels were near normal in the animals that were fed IGF-I and IGF-II.

Milk and Insulin-Like Growth Factor-I Effects upon Intestinal Metabolism

- Gene expression in mid jejunum of a key glycolytic enzyme (ICD) is markedly increased in RMS-fed rats, compared to control, with a concomitant depression of expression of enzymes responsible for local fatty acid oxidations (HADH, CPT-I).
- These findings are reversed toward control levels found in dam fed animals in rats fed IGF-I

The other thing that Rob did for us was to look at the expression of key glycolytic and beta oxidation fatty acid enzymes, because that was his real area of interest. He found that the gene expression in the mid jejunum of a key glycolytic enzyme, isocitric dehydrogenase, is one of the rate control steps for glycolysis, was markedly increased in the RMS fed rats over dam fed controls and that there was a concomitant depression of expression of the enzymes responsible for beta oxidation of fatty acids. These findings were all reversed in the animals fed IGF-I.
So our hypothesis is that in the absence of milk borne IGF-1, small intestinal metabolism shifts toward utilizing glucose as a fuel and away from fatty acid oxidation. And the intestinal glucose transporter function is also diminished. But in the presence of milk borne IGF-1, small intestinal metabolism shifts away from glycolysis toward fatty acid oxidation and intestinal glucose transport.

DR. COLLIERS: Darryl Hadsell at Baylor used transgenic mice that had very high concentrations of IGF-1 in their milk and could not demonstrate any uptake across the gut wall. Sharon Donovan used suckling pigs and found essentially the same. I am wondering why your model, the pup in the cup, where you cannulate the intestine, has such dramatic effects. What is there about that model that is different?

DR. PHILIPS: The question is what is different between these models that either show absorption or don't show absorption. I think there are a couple things that are different. Without going into the specifics, I think the length of time in which the animals are given the hormone and the absorption site that is measured. For example, we measured portal blood. We also measured peripheral blood in the acute experiments, and, again, we couldn't show any changes either. But we were able to find it in the portal blood on its way to the liver. It may be that it has more of an effect on the liver than anything else. I really don't know. But I think the length of time and the dose that was used is also quite important. And I think when we put all these things together, we may be able to figure it out and tease apart the differences.