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Discussion

DR. R. SCOTT JONES (Charlottesville, Virginia): Thank you, Dr. Wells, Dr. Copeland, Ladies, and Gentlemen.

I'd like to compliment Dr. Evers and his associates on this fine piece of experimental laboratory work. Basically, they have demonstrated that the two agents investigated altered cell growth and proliferation in liver cancer cells in culture and went on to analyze and to provide additional information so that we could understand how the agents worked.

I have two questions. One is to ask Dr. Evers if he would give us perhaps a little bit better or more complete description of the cells that he used in this particular study. As you know, most liver

cancers worldwide are produced by viruses, especially hepatitis B and also hepatitis C. But there are also hepatocyte or carcinomas that occur without evidence of viral infection, and they are probably caused by chemical carcinogens. And it would be interesting to know whether the studies that he carried out were done on viral induced cancers or chemical carcinogenic cancers.

The other question I'd like to ask is certainly obvious to all of us in this room, and that is to ask whether the agents under investigation do in fact have potential for administration to patients with hepatocellular carcinoma? And if you could spend just a moment telling us about what is known, if anything, about its route of administration, tolerance and some of the insights we'd have to have to know whether this will be useful as a therapeutic agent.

I thought this was a splendid piece of work. I had an opportunity to read the manuscript, and it is extremely well-done, well-written work, well conceived, and executed. And, again, I will compliment Dr. Evers and his associates.

Thank you. [Applause]

DR. KIRBY I. BLAND (Providence, Rhode Island): President Wells, Secretary Copeland, Fellows, and Guests. Like Dr. Jones, I can only be very complimentary of Dr. Evers and his group at Galveston for bringing this very important scientific observation to the membership. This is a well-written paper, contains a wealth of scientific data that he didn't even have time to present.

I have a number of questions as well.

As indicated, Mark, in one of your figures, the effects of differentiation and apoptosis were much more pronounced in the HepG2 variant. Interestingly, the HepG2 variant is a hepatoblastoma line which in most cell models is a less aggressive model than is the HepG3. And there were dramatic changes in both cell differentiation, which was quite different from the HepG3, and I would like to ask the authors: Would they care to speculate as to why therapy with 5-azaC produced marked morphologic changes actually for both the HepG2 as well as the more aggressive cell line HepG3?

The important but, I think, perhaps unanswerable question of this study is why butyrate did not induce changes in cell morphology for either cell line relative to the actions of that we saw with 5-azaC? Moreover, I am somewhat disappointed, and I am sure you are as well, that the combination of the agents were not more effective than either agent alone for inducing apoptosis or an inhibition of cell growth. The treatment with both of these therapies resulted in increased Bik mRNA protein, which suggests a possible role for this gene in apoptosis. Notwithstanding, though, the Bik protein expression could not be detected, which argues against this as an important process.

So the question I would have here is, since you have indicated there was no apparent change in the mRNA expression of Bcla-2 protein, suggesting this actually occurs at a posttranslational or a translational level, do you conclude that therapy 2 5-azaC, is it possible that the resultant decrease in the Bcla2 actually contributes to apoptosis?

The third question I would have is could you extrapolate this decrease in expression of Bcl xl mRNA actually occurs with simultaneous loss or static expression of Bax mRNA protein as well?

And although we have seen a number of changes, you have demonstrated an expression of the anti-apoptotic genes and the pro-apoptotic genes following therapy, each play a major role for differentiation in apoptosis after 5-azaC and butyrate therapy. So my question would be, of the genes that you have actually studied, which do you actually think influence this process? Thus, which putative genes do you anticipate to play major roles in cell differentiation and apoptosis?

And the final question, Mark, do you and your co-authors plan to do *in vivo* studies in transgenic murine models which may further elucidate these molecular mechanisms?

It's a very important paper. I again congratulate the authors, and I thank the Association for the privilege of the floor. [Applause]

DR. MARSHALL MCLEAN URIST (Birmingham, Alabama): Dr. Wells, Dr. Copeland, Members, and Guests. I, too, would like to congratulate Dr. Evers and his colleagues on an extremely well-done study and very well written manuscript. Many of the important questions have already been asked.

I'd like to focus on what is the effect of these drugs, and have you looked at them in a pharmaco-kinetic sense? That is, have you done dose response curves?

You reference in your paper the fact that there are variable results in different tumor systems reported in various laboratories. And these seem to show different culture times for the cells, different concentrations of the drug. The longer you use the drug, the higher the concentration, the more variable and certainly more profound the effect. Is this a reversible reaction? If you culture the drug with the cells and then wash it off and reculture, do you see the same kind of effect as a drug irreversibly bound to the DNA in this process?

And the other question relates to when you do this study, are there cells that are killed in this process? That is, you illustrate that there are cell-cycle arrests, there are living cells remaining. What's the nature of the cells that are killed by the exposure to the drug, if there are any?

I'd like to thank the Association for the privilege of the floor and, certainly, thank the authors for the privilege of reviewing their manuscript. [Applause]

DR. B. MARK EVERS (Closing Discussion): I'd like to thank the discussants for their thoughtful comments and would like to briefly address their questions.

I have to say that this was completely a side project from the laboratory. We are very much interested in differentiation and using these agents and looking at differentiation, but in the process of looking at differentiation, it became apparent that we could no longer not look at apoptosis, since the processes go hand in hand. And since I had a Chinese fellow surgeon in the laboratory that — in China, these cancers are quite endemic, and he wanted to take this as a side project. And, of course, I said this would never work and, you know, why don't you drop it. But the good fellows will then take that as a challenge in order to prove the boss wrong, which he did. And he came out with some interesting results, and I'd like to address some of the questions.

As far as a more complete description of the cells used, Dr. Jones asked about this. The cell lines that we have used are the standard ATCC cell lines. They are human cell lines. Hep3B is a human hepatoma cell line. I think it was taken from — I'm not sure how old the patient was. But HepG2 was taken from a 15-year-old patient and establishes a cell line. It's a hepatoblastoma. And as far looking at hepatitis B, both of these cell lines have been analyzed, and HepG2 is positive for hepatitis B, and Hep3B is apparently not.

The potential for administration of these agents, 5-azaC has

been used in various treatment trials with leukemias. I am not aware of sodium butyrate being used as far as clinical studies. It is a short-chain fatty acid which should be relatively innocuous, but it has been used in a number of experimental trials. And, for sure, there have been no trials with either of these agents in liver cancers.

Dr. Bland asked if we could speculate on some of the differences in the therapy. While we saw some more dramatic changes in HepG2 *versus* Hep3B, I think that this really gets at the mechanisms, which is what we are really interested in. There are a lot of people that have used a lot of agents, and they show apoptosis and that's where they stop. But we are more interested in trying to modulate the apoptotic pathway to actually use agents — not necessarily 5-azaC but agents like this to target specific pathways, both of the cell cycle and of the apoptotic pathway.

As far as potential differences, HepG2 cells are positive for p53 which is a tumor suppressor, which may answer some of the questions as far as differences in the two cell lines. We are currently looking at the cell cycle related protein p21, which is a cell cycle inhibitor and have some interesting preliminary data to show that p21 is increased after treatment of both of these tumor cell lines.

As far as the Bik protein, we were quite excited with our RNase protection assay. We used this, it was a multi-purposes in that we could simultaneously look at a lot of different expression levels of mRNase so that we could better hone in on which ones may be important by doing Western blots, et cetera. The Bik, which promotes apoptosis, was greatly elevated in both of these cell lines. However, when we looked at this by Western blot, we were disappointed to find very low levels of Bik protein expressed. So we are currently continuing along these lines as far as looking at various cell cycle proteins, other apoptotic proteins, in order to really dissect out the pathways better.

As far as *in vivo* studies, *in vivo* studies are planned with both liver cancers, and we are also very interested in looking at colon cancers. We have a colon cancer model that we obtained from Dr. Fiddler from Houston, which is a metastatic colon cancer cell line, and we have some preliminary data to show that there are differences in the apoptotic proteins in these cell lines. So we are very interested in those cells, looking at some of these agents *in vivo.*

Dr. Urist asked about dose response curves. We did those initially. Other people have done similar dose response curves, and the dosages that we picked were consistent with what people have shown in the literature.

As far as reversible reaction, we have not done that yet. That's interesting; it's something that we need to do, is to treat these cells, take the cells after treatment to see if they remain differentiated. Certainly, those studies are underway.

I'd like to thank the Association for the privilege of presenting this work. [Applause]