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19. ABSTRACT (Continue on reverse if necessary and identify by block number) To achieve <sup>the</sup> goal of understanding the basic mechanism of chemical inhibition of gap junctional communication (a method by which cells transfer chemical information) we have performed research on (a) validation of the fluorescence recover after photobleaching technique to study the effect of neurotoxins on cell-cell communication in living rat and human brain cells in culture; (b) development of a new <u>in vitro</u> assay to measure cell-cell communication <u>in vitro</u> , utilizing <u>scrape-loading</u> and transfer of fluorescent dyes; (c) further validation of the metabolic cooperation assay to detect tumor promoters and neurotoxins; (d) started experiments to study the possible role of protein kinase C in the mechanism of inhibiting cell-cell communication and (e) the development of a new quantitative technique to measure cell communication using flow cytometry and scrape-loading/dye transfer. The results described in this report have been communicated at several meetings. The abstracts are attached to the progress report. Reprints and preprints are also enclosed.			
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AFOSR-TR-87-0398

"The Role of Chemical Inhibition of Gap-Junctional  
Intercellular Communication in Toxicology"

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## 1. SUMMARY

A multi-cellular organism maintains its homeostatic control of cell function, within and between tissues and organs, by the process of intercellular communication. The transfer of ions and small molecules (a form of chemical information) between touching cells is mediated by the structure called a gap junction. The objective of this research project has been to develop and validate several techniques to measure gap junctional communication in mammalian and human cells grown in culture in order to study how various toxic chemicals can block this form of intercellular communication. After the first year on the project, results have been accrued to further validate the "metabolic cooperation" assay to detect several chemical tumor promoters and neurotoxins (i.e., polybrominated biphenyls, biological toxins, dieldrin). In addition, we have shown that a new laser-based technology, "fluorescent recovery after photobleaching", can be used to measure chemicals which inhibit gap junction function. More recently, we have developed two more new technologies to measure intercellular communication, namely "scrape-loading/dye transfer" and "flow cytometry-scrape-loading/dye transfer." These latter techniques are based on the principle of putting fluorescent dye molecules in living cells (as tracers) which can only go from cell to cell via gap junctions. Cells are scraped with a sharp probe in the presence of the dye, the dye enters the cell, the membrane heals trapping the dye. If the cells are able to communicate, the dye is transferred to neighboring cells. If they can not, the dye remains in the original cell. Finally, we are now starting biochemical experiments to study how chemicals, which inhibit intercellular communication, are acting.

## 2. RESEARCH OBJECTIVES

The original objectives of this proposal were to study the basic mechanisms controlling gap junctional intercellular communication in mammalian cells. To meet this objective, several specific aims were proposed:

1. To continue to validate the established "metabolic cooperation" assay to measure gap junctional communication and to test the hypothesis that chemical inhibition of intercellular communication plays a role in teratogenesis, tumor promotion and neurotoxicology.
2. To develop new assays to measure gap junctional intercellular communication.
3. To study the potential role of protein kinase C and protein phosphorylation in gap junction function.
4. To isolate gap junction proteins for the production of antibodies to be used to study gap junction function.
5. To study the possible role of oxygen-radicals and calcium in the regulation of gap junction function.

## 3. STATUS OF RESEARCH

At the end of this first year of the grant, several aims have been met and initiation of several other aims have started. Most importantly, several unexpected discoveries have been made in the development of sophisticated new techniques to measure gap junction function.

Aim 1      Validation of Metabolic Cooperation Assay to detect chemical inhibitors of gap junctional communication.

- (a) We have succeeded in demonstrating that a human teratocarcinoma metabolic cooperation assay could detect polybrominated biphenyls, a known animal liver tumor promoter. The significance of this finding is that we now have an in vitro assay potentially capable in detecting human

for publication in Fund. Appl. Toxicol.

- (b) To further validate the Chinese hamster V79 metabolic cooperation, we were able to demonstrate the assay could detect, in a dose response manner, several biological toxins (i.e., aphysiatoxin, anhydrodebromoaphysiatoxin, debromoc-physiatoxin, T2-toxin, and vomitoxin) as inhibitors of gap junctional intercellular communication at non-cytotoxic doses. The significance of these findings is that, for the first time, a cellular mechanism for the toxic effects of these biological molecules has been described. A paper has been submitted and accepted in Cell Biol. & Toxicol.
- (c) Many heavy metals are known to cause severe toxic effects in animals and humans, yet they have not been shown to be genotoxic. Using our Chinese hamster V79 metabolic cooperation assay, we have shown that arsenic acid, mercuric chloride, lead acetate and nickelous chloride all inhibited gap junctional communication at non-cytotoxic levels. A paper has been prepared and submitted for publication.

Aim 2

Development of New Assays to Detect Gap Junctional Intercellular Communication.

- (a) A significant breakthrough has been made to measure gap junctional communication. We have developed this new technique based on the principle of putting two fluorescent dyes into living cells by scraping a monolayer of cells in the presence of the dyes (which can not penetrate living cells). The cells along the wound line have their membranes tempor-

arily disrupted, allowing the dye to rush in. The membrane re-heals and traps the 2 dyes in the cell. One dye, lucifer yellow is a small molecular weight molecule which can go through gap junctions, while rhodamine-red dextran is a large molecule which can not go through gap junctions. At time zero, using a fluorescent microscope with two filters, dye molecules are seen only in the cells along the wound line. If the cells are able to communicate, in time, the lucifer yellow can diffuse away from the edge, while the rhodamine red stays at the edge. If the cells can not communicate, both dyes are found at the edge of the wound. The significance of this finding is (1) it is a inexpensive and quick assay to detect chemicals which inhibit intercellular communication; and (2) it can be used on any kind of cell type. A manuscript has been submitted and accepted in Exp. Cell Res.

- (b) In collaboration with a group at the University of Washington, we have combined the scrape-loading/dye transfer technique in the flow cytometer to come up with a very sophisticated way to quantitate gap junctional intercellular communication. The principle of the technique is, first, to scrape load fluorescent dyes into cells. Initially loaded donor cells will be identified by having both dyes in them. The recipients of dye transfer will have only lucifer yellow. The laser-assisted cell sort then can sort and quantitatively analyze the cells. We are now finishing up a series of experiments which ought to be prepared for publication in the 1/2 of the second year of the grant.

junction function.

Since many tumor promoters, growth factors and neurotransmitters have been shown to trigger their cellular effects by activating the PKC enzyme, we feel that it is important to test the hypothesis that the chemical inhibition of gap junction function could be mediated by PK-C phosphorylation of the gap junction protein (on the regulators of gap junctions).

We have recently hired Dr. Saw Yin Oh to set up the PKC assay. She and Dr. B.V. Madhukar, who is developing the electrophoretic systems to detect the phosphorylation on gap junction proteins, are now at the point where both assay systems have been calibrated in our laboratory and are working well. As of this point, preliminary experiments are being done to correlate the effect of a tumor promoter in PKC activity in the rat liver WB cell line and with the inhibition of gap junctional communication. At the rate of this initial progress, we should have an answer to our aim in the next six months.

Aim 4

Isolation of gap junction proteins for the production of antibodies.

The most direct approach to the study of chemical effects on cells is with the use of biochemical mutants and with antibodies to specific proteins. Since gap junction proteins are being implicated in the mechanism of chemical toxicity, and since antibodies to these proteins are not commercially available (nor available from other research laboratories), we are left with preparing them ourselves.



Dr. B.V. Madhukar has already started to characterize the gap junction protein electrophoretically. He now is growing up grams of cells, isolating the gap junction fraction. We will have to have freeze-fracture analysis of the pellet and then, if confirmed, antibodies will be made to this fraction. Work has progressed to the collection of the gap junction protein stage. The next six months should bring us to the point of having an antibody for molecular and biological characterization.

Aim 5

Role of oxygen-radicals and calcium in the regulation of gap junction function.

One of the current biochemical mechanisms proposed as the basis for tumor promotion (and therefore, inhibition of intercellular communication) is the chemical induction of various oxygen radical species.

We have started a series of experiments to test this hypothesis by using the xanthine/xanthine oxidase system to generate the oxygen radicals in a human keratinocyte cell system and to check if these radicals inhibit intercellular communication at non-cytotoxic doses.

Our preliminary experiments are now in progress. We must first determine the dose level of the xanthine/xanthine oxidase to use and its duration. We will use the scrape-loading/dye transfer technique to measure gap junctional communication. As soon as these parameters are determined, work should progress rapidly in the next year.

The significance of this research relates to the major importance being placed on oxygen radicals and biological toxic effects.

S. Suter, J.E. Trosko, and A. Koestner, "Fluorescence photobleaching assay of dieldrin inhibition of gap junctional intercellular communication in rat glial cells." Submitted to Fund. Appl. Toxicol.

J.E. Trosko, C. Jone, and C.C. Chang, "Inhibition of gap-junctional-mediated intercellular communication, in vitro, by aldrin, dieldrin and toxaphene: A possible cellular mechanism for their tumor-promoting and neurotoxic effects." Molecular Toxicol., in press.

C. Jone, L. Erickson, J.E. Trosko, C.C. Chang, "Effect of biological toxins on gap-junctional intercellular communication in Chinese hamster V79 cells." Cell Biol. & Toxicol., in press.

T.J. Kavanagh, C.C. Chang, and J.E. Trosko, "Effect of the polybrominated biphenyls, Firemaster BP-6, 2,2',4,4',5,5'-hexabromobiphenyl, 3,3',4,4',5,5'-hexabromobiphenyl, and 3,3',4,4'-tertrabromobiphenyl on cell-cell communication in cultured human teratocarcinoma cells." Fund. Appl. Tox. 8:127-131, 1987.

M.H. El-Fouly, J.E. Trosko and C.C. Chang, "Scrape-loading and dye transfer: A rapid and simple technique to study gap junctional intercellular communication." Exp. Cell Res. 168:422-430, 1987.

T.J. Kavanagh, P.S. Rabinovitch, M. El-Fouly, J.E. Trosko, C.C. Chang and G.M. Martin, "Use of flow cytometry and scrape-loading/dye transfer as a rapid quantitative measure of intercellular communication in vitro. In press.

R. Loch-Caruso, I.A. Corcos, and J.E. Trosko, "Inhibition of metabolic cooperation by soluble metal compounds." Submitted for publication.

J.E. Trosko and C.C. Chang, "Chemical and oncogene modulation of gap junctional intercellular communication." In: Tumor Promoters: Biological Approaches for Mechanistic Studies and Assay Systems: R. Langenbach and E. Elmore, eds., Raven Press, N.Y., in press.

J.E. Trosko, "Mechanisms of tumor promotion: Possible role of inhibited intercellular communication." Eur. J. Cancer Clinical Oncol., in press.

C. Jone, J.E. Trosko, and C.C. Chang, "Characterization of a rat liver epithelial cell line to detect inhibitors of metabolic cooperation. In Vitro, in press.

#### Professional Personnel

J.E. Trosko, Ph.D., Professor of Pediatrics/Human Development, College of Human Medicine, Center for Environmental Toxicology, Michigan State University, Principal Investigator.

B.V. Madhukar, Ph.D., Asst. Research Professor, Department of Pediatrics/Human Development.

Laurie Parker, Technician - Left for graduate school.

Saw Yin Oh, Ph.D., Research Associate, Department of Pediatrics/Human Development, (Replaces Laurie Parker).

## 6. Interactions

### A. Spoken papers.

1. M.H. El-Fouly and J.E. Trosko, "Scrape-loading and dye transfer: A rapid and simple technique for the detection of intercellular communication. Tissue Culture Assoc. Mtg., Chicago, June 4-8, 1986.
2. J.E. Trosko, C.C. Chang, M.H. El-Fouly, R. Kulkarni, and R. Gera, "Role of gap junctional communication in normal and malignant human epithelial cells. Intern. Cell and Tissue Culture Confer., Hershey, PA, Sept. 25, 1986.
3. M.H. El-Fouly, S.T. Warren, J.E. Trosko, and C.C. Chang, "Inhibition of gap junction-mediated intercellular communication in cells transfected with the human H-ras oncogene." Amer. Soc. Human Genetics Mtg., Philadelphia, PA, November 2-5, 1986.
4. J.E. Trosko (seminar), "Adaptive and nonadaptive consequences of chemical inhibition of intercellular communication." Columbia University College of Physicians and Surgeons, April 7, 1986. [Host, Dr. Carmia Borek].
5. J.E. Trosko (seminar), "Oncogenes, intercellular communication, and carcinogenesis." Dept. Pathology, New York University Medical Center, April 8, 1986. [Host, Dr. Angel Pellicer].
6. J.E. Trosko (seminar/consultant), "New methods to detect chemical inhibitors of intercellular communication." R.J. Reynolds/Nabisco Laboratory, Winston-Salem, NC, May, 1986. [Host, Dr. Dave Doolittle].
7. M.H. El-Fouly and J.E. Trosko (poster), "Role of chemical inhibition of cell-cell communication in toxicology." July 31, 1986. [Organizer, Dr. Steve Aust].
8. J.E. Trosko, "Chemical and oncogene modulation of gap junctional intercellular communication." NIEHS Conference, "Tumor Promoters: Biological approaches for mechanistic studies and assay systems." Research Triangle Park, NC, Sept. 8-10, 1986. [Organizer, Dr. R. Langenbach].
9. J.E. Trosko (symposium speaker), "The role of inhibition of DNA polymerase  $\alpha$  in DNA amplification in Chinese hamster cells." Deutscher Krebsforschungs-zentrum, Heidelberg, Germany, Oct. 24-26, 1986.

11. J.E. Trosko (seminar speaker), "Pharmacological and toxicological effects of chemical modulation of gap junction function." Dept. Pharmacology and Toxicology, M.S.U., Nov. 18, 1986.
12. J.E. Trosko (seminar speaker), "Inhibition of gap junctional communication by chemicals and oncogenes during carcinogenesis." Boston University School of Medicine, Dec. 4, 1986.
13. J.E. Trosko (seminar speaker), "Oncogenes, inhibition of intercellular communication and tumor promotion." Emory University School of Medicine, Atlanta, Dec. 12, 1986.

New Discoveries, Inventions, Patent Disclosures and Specific Applications.

- a. During this first 12 months we have made extraordinary observations leading to new, inexpensive in vitro assays to measure chemical modulation of cell-cell communication. They have the potential of being used for screening assays to detect non-genotoxic but toxic chemicals.

Additional Statements Regarding Status of Project

Because of the support of the AFOSR for this project, we have been able to re-start and re-train new personnel to work on this important area of research which our laboratory originally discovered. The significance of our research was recently highlighted by an article in Science by C.Y. Nishizuka "Studies and Perspectives of Protein Kinase C", Vol. 233:305-311, 1986, when he stated; "...and to add a new dimension essential to our understanding of cell-cell communication." [See Appendix].