Advances in Cancer Research & Therapy

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Research Article

ANALYSIS OF GENE EXPRESSION IN NORMAL AND ONCOGENE TRANSFECTED CELLS OF RAT MODEL

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Received 17 November 2014; Accepted 10 December 2014; Published 28 December 2014

ABSTRACTS

Cells in culture offer many opportunities to study the characteristics of both the normal and cancer cells. In order to understand the molecular mechanisms of cell proliferation, an attempt has been made to study the proteins which are at different phases of the cell cycle. To achieve this objective, rat embryo fibroblasts were synchronized at Go phase by serum starvation for 72h and then stimulated with serum mitogens or purified growth factors. The newly synthesized proteins were labeled with [^{35}S] methionine at different phases of the cell cycle after stimulation and the secreted proteins were analyzed by SDS- polyacrylamide gels. One of the proteins which has shown to be involved in growth regulation was purified and bioassays were carried out to determine its function. The protein 48 KDa was found to be a major compound of the extracellular matrix (ECM) whereas the protein 26 KDa was not a matrix associated protein. When the cells were arrested at G1/S boundary with hydroxyurea (HOU), high levels of protein with 45KDa protein was observed in the medium. Normal rat embro fibroblasts were transfected with myc and ras oncogenes and the transformed colonies were cultured and purified 45KDa protein inhibited the DNA synthesis of myc and ras oncogene transformed cells in which the 45KDa protein secretion was down regulated, indicating the inhibition of DNA synthesis.

KEYWORDS: Cell cycle, DNA synthesis, growth factor, Protein, Oncogene, rat embryo fibroblasts

INTRODUCTION

Cancer is a class of disease/or disorders characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distend sites by metastasis. Metastasis is defined as the stage in which cancer cells are transported through the bloodstream or lymphatic system. Cancer may affect people at all ages but risk increases with age, it was one of the leading causes of death in developed countries [1]. The unregulated growth characterize cancer is caused by damage to DNA, resulting in mutations' to genes that encode for proteins controlling cell division. Cell division or cell proliferation is a physiological process that occurs in almost all tissues and under many circumstances. Normally the balance between proliferation and programmed cell death has tightly regulated to ensure the integrity of organ and tissues.

Intense scientific effort to understand cancer disease processes and discover possible therapies, targeted therapy might be small molecular and monoclonal antibodies which play a major step in oncological treatment [2]. Targeted therapy can also involve small peptide structures which can hind to cell surface receptors or affected extracellular matrix surrounding the tumor. Radio nuclide which are attached to these peptides (e g. RGDS) eventually kill the cancer cell. Targeting of drugs that inhibit tumor growth by interfering with angiogenesis (Blood vessel formation), rather than acting directly against cancer cells the formation of new blood vessels is needed to supply the oxygen and nutrients required for tumor growth [3]. Promoting angiogenesis is crucial to tumor

development and tumor cells secrete a number of growth factor that stimulated the proliferation capillary endothelial cells resulting in the outgrowth of new capillaries into the tumor. Chemotherapy was the treatment of cancer with drugs (anticancer that can destroy cancer cells. It interferes with cell division in various possible ways which used to control tumor growth but tumor cells can develop multidrug resistance [4].

Glutathione, an endogenous intracellular thiol-containing tripeptide (L-g-glutamyl-L-cysteinyl-glycine), is an important antioxidant and has been the focus of interest in cancer chemotherapy. Under normal physiological conditions mammalian cells maintain more than 98% of glutathione in the reduced form (GSH) at intracellular concentration of 0.5 to 10 mM . GSH plays a crucial role in numerous biochemical processes including diverse bioreductive reactions, transport, protection against harmful free radicals and xenobiotics, and detoxification of metals and electrophiles [5]. Angiogenesis plays a very critical role in the development of cancer. Cancer spreads by metastasis which is the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade and grow in normal tissues elsewhere. It is this ability to spread to other tissues and organs that makes cancer a potentially life-threatening disease [6]. Recently cancer researchers involved in the study of the critical events required for the growth of a new network of blood vessels, this process of forming new blood vessels is termed angiogenesis.

In the present studies, attempts were made to monitor the loss of regulatory mechanism during transformation of normal cells by oncogenes. The interaction of oncogenes with the cellular genes was also studied in detail by molecular biological techniques. The essential approaches were transfection of normal rat embryo fibroblasts with oncogenes to study the fate of mitogen regulated proteins which play important roles in normal cell proliferation.

MATERIALS AND METHODS

Preparation of culture medium

Modified minimal essential medium of Eagle with Hank's salts and glutamine in powered form was taken directly from the pouch and dissolved in 900ml of autoclaved deionized water (Millipore) at room temperature and Sodium bicarbonate (375mg) and 50mg of gentamycin were added and mixed gently to dissolve its pH was adjusted to7.2 & the medium was sterilized by membrane filter and stored at 4°C. Medium with 20% fetal calf serum (FCS) or 10% adult bovine serum was prepared at the time of requirement. Rat embryo fibroblasts were maintained in 10% adult bovine serum containing medium and all other cell lines were propagated in 10% FCS containing medium.

Preparation of primary rat embryo fibroblasts (REF)

Primary culture of rat embryo fibroblasts was prepared according to the procedure described by [7]. The embryos were taken from the rat on the thirteenth to fifteenth day of pregnancy and processed for primary culture as described below. The pregnant rat was sacrificed by cervical dislocation and the ventral surface of the abdomen was sprayed thoroughly with 70% and swabbed with sterile cotton. The abdomen was cut open along with the mid ventral line and the embryos were exposed. The intact uteri were removed from the blood vessel connections and transferred to a petridish containing sterile phosphate buffered saline (PBS).the embryo were relieved from the uteri, washed with PBS and the head, visceral organs and appendages were removed. The remains of the embryos were washed with PBS and minced with a bent scissors. The tissue was transferred to the trypsinizing flask containing 0.25% trypsin and stirred for 30 min at 37 °C to facilitate trypsin's action in releasing individual cells from the tissue. The cell suspension was filtered through a sterile cheese cloth and the filtrate was centrifuged to pellet down the cells (500x g for10 min). The cell pellet was resuspended in 5ml of MEM (MEM- modified eagle's minimum essential medium) with 10% bovine serum and viability was checked by staining a few cells with 0.4% erythrocin B. Glass bottle of 250 cm^2 size for culturing and regular maintenance of the cells were obtained from flow laboratories. For large scale culture, roller bottles were obtained from Wheaton Company USA. Approximately $3.6x10^5$ cells in 7ml of medium were added to each culture bottle and incubated at 37 °C.

Synchronization

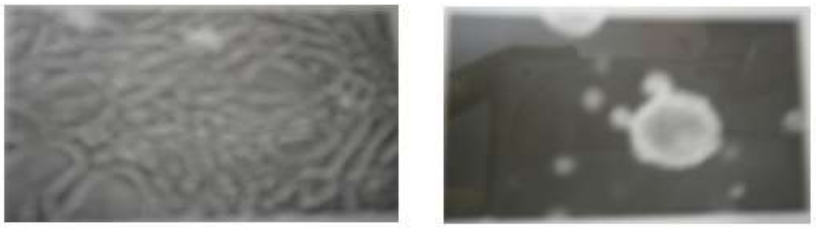
Cells were synchronized at quiescence by washing proliferating sub- confluent monolayer thrice with PBS and then placing them in medium with 0.5% serum for 48-72 hrs. These cells were designated as serum arrested cells. During serum starvation, the cells enter into Go stage of the cell cycle and they virtually stop proliferation. Serum stimulated cells or growth factor stimulated cells were obtained by releasing the serum arrested cells with 10% or 20% FCS containing medium or by growth factor addition to the medium. In another set of experiments the cells were synchronized at G1/S border by double block method by arresting cells first at quiescent condition as mentioned above and then treating with 1mM hydroxyurea (HOU).In 10% serum containing medium for another 16h. Cells arrested in G1/S border by HOU arrest were released by washing them thrice with PBS followed by incubation in medium containing 20% FCS these cells were referred to as HOU-released cells.

RESULTS AND DISCUSSION

The control of cell proliferation was regulated external growth factors and by cellular genes and gene products that respond to those growth factors. Several studies are focused on the induction of proteins which are newly synthesized in response to the external stimuli. Identification of growth factor and growth inhibitors and of genes and gene products that regulate the cell proliferate was essential to understand the molecular mechanisms of cell proliferation. When rapidly proliferating normal fibroblasts were exposed to a low serum containing medium enter into a specific resting stage known as quiescence. It is possible to make the quiescent cells re-enter the cell –cycle by increasing the serum concentration of the medium. The genes that control the transition from G0 to G1 period hold the key to the control of cell

proliferation and important biochemical regulatory events necessary for the proper initiation of cell-cell take place in this period. Several genes were induced and a number of proteins were synthesized in response to the mitogenic stimulus. In the results revealed with the secreted proteins of normal secondary rat embryo fibroblasts (REF) induced by serum mitogens, and inhibition of some of the secreted proteins upon oncogenic transformation.

In oncogene transfection studies, secondary rat embryo fibroblasts were transfected with myc and ras oncogenes by calcium phosphate co precipitation method. The cells were allowed to proliferate and transformed foci were observed 21 days after transfection. For soft agar culture myc and ras oncogene transformed cells were suspended in 0.3% agarose containing MEM with 10% FCS and then plated on a 0.6% agarose layer containing MEM and 10% FCS in petridishes. Figure 1 shows the transformed anchorage independent cells 4 weeks after plating in soft agar. One of the criteria of transformation was the growth of cells without anchorage. Transformed cells do grow in semisolid media of agar or agarose and form colonies whereas normal cells do not grow in the semisolid medium and eventually die. Rat embryo fibroblasts were transfected with myc and ras oncogenes as explained and plated in 0.3% agarose containing medium with FCS, several anchorage independent colonies were observed after three weeks as shown Fig1-b fulfilling one of the characteristics of transformed cells.



A. *Normal REF* **B.** *myc-ras transformed colony*

Figure 1*: Transformed colonies of rat embryo fibroblasts (REF)*

Legend: *Transformed colonies of rat embryo fibroblasts (REF).A: Normal REF, B: the myc-ras transformed colony in soft agar culture shows the anchorage independent cells expressed oncogene confer G418 resistance in transformed cells.*

Figure 2 shows the Geneticin 418 resistant colonies of myc and ras transfected cells. Secondary REF were transfected with myc (5µg) ras (5µg) and pSV_2 (1µg) plasmids by calcium phosphate co precipitation method. The cells were split into 1: 4, 48 h after transfected into G418 (400 µg/ml) containing medium. G418 resistant colonies appeared 3 weeks after transfection. The colonies were fixed in methanol and stained with Giema.

Figure 2: *Geneticin 418 (G418) resistant colonies of myc and ras transfected cells*

Legend: *Culture bottle shows the G418 resistant colonies 14 days after transfection*

Proliferating sub –confluent monolayers of REF were arrested in G1/S border of the cell cycle by double block method (serum starvation and HOU arrest).The cells were driven to quiescence by maintaining in 0.5% serum containing medium for 72 h and then treated with 1mM HOU in MEM containing 10% FCS for 20h. DNA synthesis was monitored by labeling the cultures with 5µci/ml of [^{3}H] thymidine for 30min at different periods after release from HOU-arrest. [^{3}H] thymidine incorporation into DNA was determined. These cells started to synthesize DNA as soon as the HOU block was removed and the synthesis reached a peak at 3h after release and then gradually declined. An inverse correlation was reported earlier between low levels of protein 45 KDa and DNA synthesis in mouse embryo fibroblasts. Figure 3 shows the protein profile of HOU arrested and released cells. Interestingly, high levels of a 45KDa protein were secreted from HOU arrested cells. A gradual decline in the of this protein at 1h and 2h and a drastic reduction at 3h after release from HOU-arrest was observed.

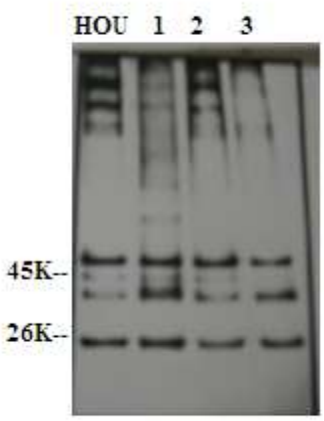


Figure 3: *Secreted proteins of HOU arrested and released cells*

Legend: *Secreted proteins of synchronized cultures of rat embryo fibroblasts. Sub-confluent cultures were synchronized by serum deprivation followed by Hydroxyurea treatment. The cells were labeled with [^{35}S] methionine after release from HOU arrest for 30 min and then chased for 30 min in serum from MEM. Proteins in the chase medium were analyzed by SDS PAGE followed by fluorography. HOU, secreted proteins from HOU arrested cells. The numbers 1, 2 and 3 above lanes indicate the time in hours after HOU release*.

The result showed that serum stimulated REF also secreted a protein of 45KDa and it was super induced by cycloheximide treatement in quiescent cells. In HOU –arrested cells with negligible DNA synthesis, the 45KDa protein level was more than that found in HOU- released cells. The level of this protein gradually declined when the cells entered into S phase (Figure 3).These results suggested an inverse correlation between the level of the 45KDa protein and DNA synthesis. It appears that there was an autocrine regulation of DNA synthesis by the 45KDa protein in which high levels of the protein in the medium turn-off DNA synthesis subsequently.

Secreted proteins of normal and transformed cells were known to manifest in a myriad of changes, the level of the secreted inhibitory activity was determined in transformed cells. The transformed cells were labeled with [^{35}S] methionine and the secreted proteins were isolated and analyzed by SDS- PAGE. Figure 4 shows the protein prolife of normal and myc –ras transformed cells in which a reduction in the level of the protein 45 KDa was evident. Another secreted protein of molecular weight 48 KDa which was identified as plasminogen activator inhibitor -1 also depicted reduced levels in myc-ras transformed cells. Since the transformed cells showed reduced levels of 45KDa protein has DNA synthesis inhibitory activity on myc-ras transformed cells. It was evident from the studies presented here that the 45KDa protein is a negative regulatory protein which inhibits DNA synthesis. The myc-ras transformed secondary REF exhibited drastic reduction in the level of the 45KDa secreted protein Figure 4 suggested that this protein may interfere with rapid proliferation of potential transformed cells. It was interesting to note that exogenous addition of 45KDa protein to the same transformed cells inhibits DNA synthesis.

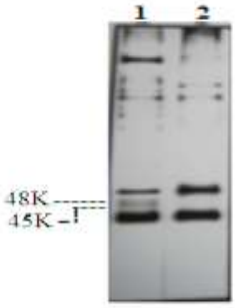


Figure 4: *Secreted proteins of Normal and Oncogene transformed cells*

Legend: *Normal and oncogene (myc and ras) transformed REF were labeled with [^{35}S] methionine for 30min and chased for 30 min, the protein in the chase medium were electrophoresed in SDS-PAGE and fluorographed. Lane1: Normal Lane 2: myc and ras transformed cells.*

Immunoprecipitation of 48 KDa secreted protein with antibodies to plasminogen activator inhibitor-1 (PAI-1). Figure 5 shows quiescent monolayers were stimulated with 20% FCS for 3h and then labeled [^{35}S] methionine for 30min and chased for 30 min. The secreted proteins in the chase medium were concentrated and immunoprecipitated. The immunological assays indicate that the serum stimulated cell secreted 48 KDa protein and extracellular matrix associated 48 KDa protein were related to PAI-1 (Figure 5). Based on the results of the absence of this protein in quiescent cell conditioned medium, it was concluded that the PAI-1related48 KDa protein is induced upon mitogenic stimulation and secreted into the conditioned medium of stimulated cells. Exposure of quiescent cells to fetal calf serum was shown to result in the growth medium via extracellular matrix in mouse embryo fibroblasts [8].

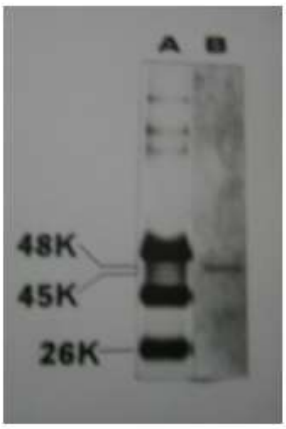


Figure 5: *Immunoprecipitation secreted protein 48 KDa with antibodies to PAI-1*

Legend:

Lane A: Labeled proteins of conditioned medium of serum – stimulated cells.

Lane B: Conditioned medium proteins of serum –stimulated cells immunoprcipitated with PAI-1 antibodies.

The association of plasmingn activator expression with transformed phenotype suggested the possibility that plasminogen activators of plasminogen may be involved in tumor growth, tissue degradation, tumor invasion and metastasis causing connective tissue destruction. The disappearance of secreted PAI-1 in myc and ras oncogene transformed cells may be results an increase in the activity of PAs which destroy the anchorage thus enabling the transformed cells to lose adhesion. Since malignant cell growth can lead to active matrix degradation both in vivo, and in vitro, it was accompanied by the inhibition of PAI-1 in the transformed cells. It would be interesting to study the proposed inhibitory effect of PAI-1 synthesis on tumorigenicity by monitoring the effect of expression of PAI-1 gene in transformed cells.

CONCLUSION

Cells in culture offer many opportunities to study the characteristics of both the normal and cancer cells. In order to understand the molecular mechanisms of cell proliferation, an attempt has been made to study the proteins which were induced at different phases of the cell cycle. To achieve this objective, rat embryo fibroblasts were synchronized at Go phase by serum starvation for 72h and then stimulated with serum mitogens or purified growth factors. The newly synthesized proteins were labeled with [^{35}S] at different phases of the cell cycle after stimulation and the secreted proteins were analyzed by electrophoresis in SDS poly acrylamide gels followed by fluorography. One of the proteins was shown to be involved in growth regulation, the 48KDa protein was found to be a major component of the ECM whereas the 26KDa protein was not a matrix associated protein. The two proteins (48KDa and 45KDa) identified here may play important roles in cell proliferation. The 48KDa protein identified as PAI-1 was shown to be trans located from the membrane to the medium upon mitogenic stimuli, and the 45KDa protein as DNA synthesis inhibitor disappear upon transformation. The oncogene transfected cells showed down regulation of both 48KDa and 45KDa proteins suggested that transformed cells shut off the synthesis of these proteins, it was speculated that ras oncogene activates a growth related gene.

REFERENCES

[1] Erculj, N., Kovac, V.,Hmeljak, J., Dolzan, V. 2012. The influence of platinum pathway polymorphisms on the outcome in patients with malignant mesothelioma. Annals of Oncology. 23, 961–967.

[2] Khrunin, AV., Moisseev, A.,Gorbunova,V., Limborska, S. 2009. Genetic polymorphisms and the efficacy and toxicity of cisplatin-based chemotherapy in ovarian cancer patients. Pharmacogenomics J. 10, 54–61.

[3] Caronia, D., Patino-Garcia, A., and Milne, R.L. 2009. Common variations in ERCC2 are associated with response to cisplatin chemotherapy and clinical outcome in osteosarcoma patients. Pharmacogenomics J. 9, 347–353.

[4] Zucali, PA.,Giovannetti, E., Destro, A. 2011. Thymidylate synthase and excision repair cross-complementing group-1 as predictors of responsiveness in mesothelioma patients treated with pemetrexed/carboplatin. Clin Cancer Res .17,2581–2590.

[5] Workman, P., Aboagye, EO., Balkwill, F., Balmain, A., Bruder, G., Chaplin, D.J.2010.Guidelines for the welfare and use of animals in cancer research. Br J Cancer.102,1555–77.

[6] Dieckmann, KP., Struss, WJ., Budde, U.2011. Evidence for acute vascular toxicity of cisplatin-based chemotherapy in patients with germ cell tumour. Anticancer Res. 31,4501–505.

[7] Gwatkin, RBL. 1973. In tissue culture: Methods and applications. ed. Krusse PF and Patterson MKacademic press USA. 3-5.

[8] Srinivas, S ., Nagashunmugam, T., Shanmugam, G. 1990. J.Biosciences. 15, 351-359.