Sequential enhanced tyrosine phosphorylation during progressive malachite green induced malignant transformation of Syrian hamster embryo cells in culture is associated with no change in the activity levels of tyrosine phosphatases

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Malachite green (MG) consisting green crystals with a metallic lustre is extremely soluble in water and is highly cytotoxic to mammalian cells and also acts as a liver tumour promoter. In view of its industrial importance and possible exposure to human beings, MG poses a potential environmental health hazard. We have earlier reported that MG induces malignant transformation in Syrian hamster embryo (SHE) cells. Since tyrosine phosphorylation and dephosphorylation reactions are known to play critical roles during normal and abnormal cellular proliferation, in this study we have studied the tyrosine phosphorylation, tyrosine phosphorylated proteins and protein tyrosine phosphatases in malignant transformed cells and during sequential development of cellular transformation by MG compared to control cells. The present investigation shows that enhanced tyrosine phosphorylation and tyrosine phosphorylated proteins associated with the static levels of tyrosine protein phosphatases may probably contribute to the abnormal cellular proliferation during malignant transformation of SHE cells by MG.

Presence of several nonpermitted food colouring agents such as Malachite Green, Orange II, Metanil Yellow, and Rhodamine B in food materials have been previously reported from food preparations sold in the market. Some of these additives and industrial colours are dyes containing amino or methyl groups which either form nitronium ions or carbonium ions which can yield DNA adducts thus increasing risk of cancer. One such compound is Malachite Green (MG), a triarylmethane dye. It has been identified as harmful by WHO/FAO expert committee and is toxicologically classified under category III. It is also used extensively for dyeing cotton, wool, jute, leather, as a laboratory reagent and also as topical antiseptic. Despite the deleterious effects, there is continuous occupational exposure of MG to workers in printing and textiles industries and it is also reported to be present in an unscrupulous way in juices and other eatables consumed by people from lower socio-economic strata especially school children. In view of its multipurpose utility, it poses a great health hazard and is of considerable environmental concern. However, despite its known harmful effects, there is still a big lacuna in the information on cytotoxicity, carcinogenicity and the mechanism of action of MG.

We have previously reported that MG is extremely cytotoxic to mammalian cells in culture and also acts as a liver tumour promoter. Furthermore, treatment of SHE cells (Syrian hamster embryo) with MG results in an induction of monooxygenase system, lipid peroxidation, and catalase activity in a dose dependent manner indicating the possible formation of reactive free radicals. The free radical generation by MG in SHE cells was confirmed using ESR spectroscopy and also by detecting the DNA damage in the form of single strand breaks. There is substantial evidence showing the involvement of free radicals in toxicity, ageing, carcinogenicity etc. Accordingly, it is likely that the exposure of MG to SHE cells may result in the oxidative damage due to free radical formation, which is likely to induce cellular transformation. In view of the above assumption, we have studied the malignant transforming potential of MG using SHE cells as the model system and obtained MG transformed cell lines from these cells.

Phosphorylation-dephosphorylation reactions at tyrosine residues have been implicated to play an important role in cell division, differentiation, cell cycle regulation, transformation, development and memory. This post translational modification is
dependent on two classes of enzymes- protein tyrosine kinases and protein tyrosine phosphatases and their proper ratio is highly essential for maintaining the homeostatic balance in cell system\textsuperscript{10-12}. Tumour promoters and transforming agents either by activating the kinases or inhibiting the phosphatases bring about an aberration in protein phosphorylation leading to unrestrained cell growth and expression of malignant phenotype\textsuperscript{13}. The link between tyrosine phosphorylation and malignant transformation is well established and the products of several retroviral transforming genes have been shown to possess tyrosine kinase activity, which is necessary for transformation\textsuperscript{14}. In view of this we have made an attempt to study the tyrosine phosphorylation and dephosphorylation in MG transformed cells and wish to report in this paper that sequential enhanced tyrosine phosphorylation during progressive malachite green induced malignant transformation of Syrian hamster cells in culture is associated with no change in the activity levels of tyrosine phosphatases.

Materials and Methods

Cells and culture conditions—Primary SHE cultures were set up from 10-14 day gestation fetuses collected aseptically. Cells were grown in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10\% heat inactivated fetal calf serum (GIBCO) and Gentamycin (50 \(\mu\)g/ml) and maintained at 37\textdegree C in 5\% CO\textsubscript{2} atmosphere. For secondary cultures, cells were transferred by trypsinisation with 0.25\% trypsin\textsuperscript{6}.

MG treatment—Stock MG solution was prepared fresh in sterile saline, passed through millipore membrane filter, diluted with saline and added to the culture dishes at appropriate concentrations. Control cultures received only sterile saline at the same volume usually less than 50 \(\mu\)l and at this level had no effect on cell growth.

Morphological transformation—Transformation assays were carried out as per the method essentially described by Reznikoff et al\textsuperscript{15}. Cells were seeded in 60 mm petridishes (20 petridishes per group) at a density of 5000. Five days after seeding, cells were treated with MG at a concentration of 0.05 \(\mu\)g/ml based on cytotoxicity studies carried out. One week later, media change was given. Subsequently, media was changed at weekly intervals and at 6th week, dishes were fixed with methanol, stained with Giemsa and morphologically transformed foci were counted.

In all the assays, the solvent in which MG was dissolved i.e. saline served as negative control. Transformed foci were classified as type II and type III using the morphological criteria described by Reznikoff et al\textsuperscript{15}. Some of these MG transformed type III foci were utilised for establishing immortal cell lines. These immortal cell lines showed formation of colonies in soft-agar and induced tumours in nude mice which were found transplantable\textsuperscript{9}. These cells were utilised for studies on phosphorylation and dephosphorylation. At the time of experiments, transformed cells were in Passage No.55-65 and corresponding controls in Passage No.3-4. For sequential analysis studies fresh primary cultures were utilised.

Preparation of cell lysates for kinase and phosphatase assays—Cells were harvested by scrapping, pelleted and sonicated in the dilution buffer (20 mM Tris pH 7.1, 150 mM NaCl, 5 mM EDTA, 2 \(\mu\)g/ml each of aprotinin, leupeptin, pepstatin, 100 \(\mu\)M Na-orthovanadate, and \(\beta\)-Mercaptoethanol at 0.025\% concentration). The sonicated extract was centrifuged at 10,000 rpm for 20 min. The protein content of the supernatant was estimated by Lowry's method\textsuperscript{16} and equal amount of protein was utilised for all the assays.

Assay for tyrosine specific protein kinase activity—Tyrosine specific protein kinase activity was assayed by using poly (Glu,Tyr), a random copolymer of glutamic acid and tyrosine in the ratio of 4:1 as substrate. The assay mixture contained 100 mM Tris pH 7.5, 25 mM MgCl\textsubscript{2}, 1mM vanadate, 50 mM DTT, 2 \(\mu\)g/ml poly (Glu,Tyr) and 10 \(\mu\)M (\(\gamma\)-\textsuperscript{32}P) ATP and the sample of lysate preparation in the total volume of 25 \(\mu\)l. The reaction was started by the addition of ATP and incubating the mixture at 30\textdegree C for 15 min. 2X sample buffer was added to stop the reaction and the lysates were electrophoresed on a 10\% SDS-PAGE\textsuperscript{17}. The gels were stained, dried, and autoradiographed. The gel lanes were cut and counted. Counts were expressed in cpm/10 min. For each sample, appropriate controls were kept.

Endogenous phosphorylation—Samples were incubated in a total volume of 25 \(\mu\)l containing 20 \(\mu\)m (\(\gamma\)-\textsuperscript{32}P) ATP in the assay buffer as described above but without the substrate. Incubation was carried out at 25\textdegree C for 10 min. The reaction was stopped by the addition of an equal volume of 2X sample buffer and samples were analysed by SDS-PAGE followed by
I autoradiography and rest of the procedure was same as described above.

**Western blotting of control/transformed cell lysate for phosphotyrosine**—Control and transformed cells were harvested by scraping, pelleted and lysed in lysis buffer (50 mM Tris pH 7.4, 5 mM EDTA, 10 mM Na pyrophosphate, 0.2% SDS). Triton-X-100 (2%), 2 μg/ml each of aprotinin, leupeptin, and pepstatin were added to the lysates. The samples were boiled for 10 min and treated with DNase (0.5 μg/ml). Cellular debris was removed by centrifugation at 10,000 g for 5 min. 75-100 μg of the proteins were electrophoresed on a 10% SDS-PAGE and transferred to nitrocellulose membrane. Transfers were stained with Ponceau S to confirm the presence of equal amount of proteins in each lane. 5% skimmed milk was used for blocking for 1½ hr at 55°C. Monoclonal phosphotyrosine antibody (SIGMA) at 1:700 dilution was added for 3 hr at room temperature followed by overnight incubation at 4°C. The binding of the antibody to the protein was detected using GAM conjugated to peroxidase (1:8000) using ECL Amersham kit.

**Phosphatase assay**—PTPase assay was based on measurements of the decrease in the amounts of phosphorylated ³²P-labelled poly (Glu,Tyr) 4:1, a random copolymer of glutamic acid and tyrosine as substrate. The-cell lysate was incubated at 30°C for 10 min in assay buffer (0.1 M Tris, pH 8.0, 0.2 M NaCl₂) containing 0.3-2.0 μm ³²P-labelled poly (Glu,Tyr) 4:1, in a total volume of 50 μl. The incubation was stopped by adding 200 μl of cold 10% trichloroacetic acid followed by the addition of 20 μl of bovine serum albumin (20 mg/ml). After keeping at 4°C for 15 min, the tubes were centrifuged for 2 min in an eppendorf centrifuge. The trichloroacetic acid soluble material (200 μl) was counted directly for radioactivity. The percentage decrease in trichloroacetic acid precipitable ³²P poly (Glu,Tyr) (4:1) was taken as the measure of PTP activity.

**Preparation of ³²P labelled substrate**—³²P labelled poly (Glu,Tyr) 4:1 was prepared essentially as described earlier. Briefly, the polymer (1 mg) was incubated with 0.1 m M (γ⁻³²P) ATP in 100 mM Tris buffer containing 50 mM of MgCl₂, 1 mM vanadate, and 50 mM DTT and 10 μg of tyrosine kinase (TK-1) purified from rat spleen. After incubating for 30 min at 30°C, another aliquot of kinase was added, and the incubation continued for an additional 30 min. The reaction was stopped by adding trichloroacetic acid (20% final concentration). After 15 min at 0°C, it was centrifuged for 2 min in an eppendorf centrifuge, washed once with 10% TCA, and dissolved in 0.5 ml of 1 M Tris (pH 8), and dialysed against 10 mM Tris pH 8.0 overnight with two changes. The concentration of ³²P labelled substrate was determined from the specific activity of the (γ⁻³²P) ATP used for the phosphorylation of the substrate.

**Sequential analysis studies of tyrosine kinase and tyrosine phosphatase activity**—Cells were exposed to MG at 0.05 μg/ml concentration for a period of 1 week as described in transformation procedure. For sequential analysis studies, cells were utilised at different time periods following one week of MG exposure. PTK and PTP activity were assayed as described above.

**Results**

**Tyrosine specific protein kinase activity**—Control (Passage No. 3-4) and transformed cells (Passage No 55-65) were assayed for the total tyrosine kinase activity, using poly (Glu,Tyr) in the ratio of 4:1 as the substrate. Transformed cell lysate consistently showed a 2 to 3-fold higher activity than the controls (Fig.1) at all time periods tested indicating that total tyrosine phosphorylation probably by more than one

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**Fig.1**—Time course kinetic study of tyrosine phosphorylation in normal and MG transformed S1E cells. Experimental details are given under Materials and Methods. Each value represents mean ± SE of three values.
kinase is elevated in transformed cells compared to controls.

*Endogenous phosphorylation*—Tyrosine kinases are capable of not only phosphorylating other proteins but also specific tyrosine residues on themselves and autophosphorylation can be either an intermolecular or intramolecular interaction. Accordingly to test whether increased tyrosine kinase activity in transformed cells was reflected by a greater amount or a greater variety of tyrosine kinases and/or their substrates, an *in vitro* endogenous phosphorylation was carried out without the addition of poly (Glu, Tyr) followed by alkaline treatment. Again the endogenously phosphorylated gel showed an increase of 189% of the phosphorylated proteins in transformed cells compared to control cells, thus confirming the observations using poly (Glu, Tyr) showing an increased tyrosine phosphorylation in transformed cells as compared to the controls.

*Western blotting of control/transformed cell lysate for phosphotyrosine*—Control and transformed cell lysates were immunoblotted for tyrosine phosphorylated proteins using phosphotyrosine monoclonal antibodies. The pattern of bands showed both qualitative and quantitative differences in phosphotyrosine containing proteins between control and transformed cells. Transformed group (Fig. 2) showed more intense bands in the high molecular weight range.

*Phosphatase assay*—In order to see if the increased tyrosine kinase activity is associated with the corresponding decrease in the total tyrosine phosphatase levels, we tested for the status of tyrosine phosphatases in control and transformed cells using $^{32}\text{P}$ poly (Glu, Tyr) as the synthetic substrate at different concentrations of cell lysates. The analysis of control and transformed cell lysates revealed no difference in the total phosphatase levels in both the groups (Fig. 3).

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**Fig. 2**—Western blotting of tyrosine phosphorylated proteins in normal and MG transformed SHE cells. Experimental details are given under Materials and Methods. Lane 1, Control; Lane 2, Transformed.

**Fig. 3**—Kinetic study of tyrosine phosphatase at different protein concentrations in normal and MG transformed SHE cells. Experimental details are given under Materials and Methods. Each value represents mean ± SE of four values.
Sequential analysis of tyrosine kinase and tyrosine phosphatase activities during different stages of transformation—In order to understand the possible role of tyrosine phosphorylation at different stages of transformation and its possible functional relevance to transformation we have studied the levels of PTK and PTP at different stages of transformation. Using the standard transformation protocol as described in Materials and Methods, we have assayed TK activity at different time periods following exposure to MG for 1 week. An increase of 8, 25, 43% in tyrosine phosphorylation levels was observed at 1, 3, 5 weeks exposure to MG respectively indicating a sequential enhancement in total tyrosine kinase activity. However no change in the total phosphatase levels was observed at different stages of transformation at 1, 2, 3, 4, and 5 weeks exposure to MG, thereby indicating no sequential change in phosphatase levels following transformation. (Fig.4).

Discussion
An impressive body of evidence supports the idea that malignant transformation is due to multiple genetic alterations. Of the implicated genes, a large proportion code for proteins with tyrosine kinase activity. The evidence implicating protein tyrosine kinases as critical regulators of cell proliferation has come mainly from two lines of enquiry - the first one from analysis of a variety of transforming retroviruses which have shown that a considerable proportion have transforming genes that code for protein tyrosine kinases and the second one on growth factor receptors which have revealed that the effector domains of a number of these proteins have intrinsic tyrosine kinase activity, indicating the importance of tyrosine phosphorylation in the control of normal and abnormal cellular proliferation. In order to understand the role of phosphorylation and dephosphorylation reactions during malignant transformation we have studied both tyrosine phosphorylation and dephosphorylation during MG induced transformation.

The analysis of cell lysates from control and transformed group for their total TK activity using a synthetic substrate poly (Glu, Tyr) 4:1, revealed a 2-3-fold increase in the transformed group as compared to the control group. To see whether this was reflected in the phosphorylation status of cellular proteins, endogenous phosphorylation reactions were carried out and the increased endogenous tyrosine phosphorylation in the transformed cells supported the results of TK activity assay using poly (Glu, Tyr). Immunoblotting studies also confirmed the enhanced presence of tyrosine phosphorylated proteins in transformed cells compared to controls. To see whether this increase in tyrosine phosphorylation is associated with a decrease in tyrosine dephosphorylation reactions we have studied the protein tyrosine phosphatases in transformed as well as in control cells. The analysis of the cell lysates from transformed and control group for their total PTPase activity using 32P labelled poly (Glu, Tyr) as substrate revealed no difference in their levels. The sequential analysis studies carried out at different stages of transformation following one week of MG exposure also showed a sequential enhancement in the PTK activity with increasing time period of transformation. However no change was observed in the PTP levels following transformation.

A number of studies have indicated a potential role for serine/threonine specific PP1 and PP2 A in the regulation of cell growth. The observations that okadaic acid is a powerful tumor promoter and a specific inhibitor of PP1 and PP2 A has led to the suggestion that these two enzymes function as tumor suppressors in mammalian cells. Protein tyrosine
phosphatases exist as two classes, soluble cytoplasmic and membrane bound and the turnover number of cytosolic protein tyrosine phosphatases greatly exceeds those of protein tyrosine kinases. This explains the low steady state levels of tyrosine-phosphorylated proteins during normal cellular proliferation. It is likely that protein tyrosine phosphatases may act as growth inhibitory or tumour suppressor proteins by counteracting the growth stimulatory effects of activated protein tyrosine kinases. It is interesting to note that in our study there is an increase in tyrosine phosphorylation and as well as tyrosine phosphorylated proteins in transformed cells compared to control, while no change in the tyrosine phosphatase activity levels either in the malignantly transformed cells or during sequential development of cellular transformation. These results suggest that during malignant transformation which is associated with abnormal cellular proliferation the activated protein tyrosine kinases contribute additionally to the abnormal cellular proliferation by growth stimulatory effects while there is no change in the protein tyrosine phosphatases, indicating no additional inhibitor or suppressor effects. In summary, the present investigation provides evidence that static protein tyrosine phosphatases associated with enhanced protein tyrosine phosphorylation and tyrosine phosphorylated proteins may contribute to the abnormal cell proliferation during malignant transformation. Studies are in progress to identify the specific protein tyrosine kinases involved in the increased tyrosine phosphorylation.

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