CHAPTER VI

HISTOLOGICAL STUDIES
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Histological studies were conducted as a part of the present investigation to evaluate the carcinogenic changes brought about by the hepato-carcinogen-azoearmine and subsequent revival effect of vitamin C administration. During the onset of carcinogenic induction, the changes in the histological structure determines the proliferative activity of the cells. The gross structural changes in the histological picture with loss of normal cell patterns and gradual increase in the population of proliferative, carcinomatous cell may be taken as an index for the advancement of carcinogenesis in the experimental animal.

The carcinogenic growth of the liver due to the administration of azo-dye produced carcinomatous changes associated with occurrence of few nodules. The nodule masses were bulky and soft and frequently showed necrosis. Usually it was seen to be associated with cirrhosis of the portal type. It appears that the cirrhosis is the primary lesion, the tumour arising from the hyperplastic nodules. Invasion of the veins was frequent. As per histological studies tumour cells were giant and multinucleated cells were not rare. The main histological changes in the early stage of treatment was indicated by occurrence of few enlarged and vacuolar cells showing marked granular deposit in cytoplasm. The size of the cells were also markedly increased, the cell also showed profuse vacuolation. Gradually the growth of this type of cell by the side of arteries and veins showed gradual invasion in the inner part of the liver tissue.
With the continuation of the treatment the histological picture exhibits a process of cell proliferation and cell loss side by side and ultimately produced the advancement of the carcinogenic changes with formation of number of nodules as a result of prolonged carcinogen administration, with subsequent loss of normal histopattern of the liver. In the later period, some of the neoplastic cells were found to be infiltrated inside the blood vessels which indicated the beginning of metastatic changes.

The present experiment is mainly concerned with the induction of carcinogenic changes in the liver histology and the possible revival of carcinogenic changes with oral administration of vitamin C.

1. CONTROL SET OF EXPERIMENT:

Histological study of liver from the control set of the experiment showed a more or less similar picture to one another throughout the experimental period. Haemotoxylin and eosin staining of the liver cells of the control guinea-pigs showed the presence of hexagonal cells with the prominent nucleus in every cell. The liver structure represents some hexagonal lobules composed of cords of identical cells centered around a terminal hepatic vein, known therefore as the central vein. The presence of innumerable bile ducts gives it a hexagonal shape. Arterioles and venuoles were found throughout the organ. The cytoplasm was uniform with different cellular organelles. The nucleus stained deeply (Plate No. 30) and was surrounded
PLATE No. 30: Microphotograph showing the histological picture of the normal liver cell of guinea-pig.

PLATE No. 31: Microphotograph showing loss of cellular adhesiveness and the gradual disappearance of cementing material after 14 days of carcinogen treatment.

PLATE No. 32: Microphotograph showing deterioration of the cellular architecture and the partial destruction of the cells adjacent to the arterioles and venules.
by a band of cytoplasm. Bile canaliculi were clear. A uniformity in the size of the cells was a marked feature. Arterioles and venules took the same uniform staining as did other components of the organ. The cells were found to radiate from a common centre in the form of definite cord like structures.

2. CARCINOGEN TREATED SET:

The present experiment demonstrated that the cellular structure of the liver is drastically changed after the administration of the azo-dye-azocarmine. These liver sections when stained in haemotoxylin and eosin showed a number of variations to that of the control set of the experiment.

Studies were carried out in the histology of the liver after seven days of carcinogen treatment. It revealed a loss of compactness of the hepatic cells in comparison to that of the control. Nuclear changes were not remarkable. The structure of the cytoplasm was more or less comparable to that of the control. But the adhesiveness between the hepatic cells was lost.

With the advancement of carcinogenic treatment, the liver cells showed a gradual increase in the loss of cellular adhesiveness. The cementing matrix gradually disappeared (Plate No. 31) and the hexagonal cells became isolated from one another. Enlargement of the nuclear size was recorded. The distinction in the staining of the nucleus and the cytoplasm which was marked in the control set was reduced with a partial loss of transparency.
PLATE No. 33: Microphotograph showing the appearance of vacuolar spaces in the liver section after the administration of carcinogen.

PLATE No. 34: Microphotograph showing the disintegration of the blood vessels due to the administration of carcinogen.

PLATE No. 35: Microphotograph showing the invasion of the liver cells from the venuole.
Liver cells after 21 days of treatment with chemical carcinogen showed a further deterioration of the normal histological structures. Along with the loss of cellular adhesiveness these cells were marked with the appearance of granular deposition in the cell cytoplasm. The nuclear size and cell size was larger to that of the control and that of the 14 days treated set.

Continuation of the treatment with azocarmine for a period of 28 days further deteriorated the cellular architecture. This liver section was marked by a partial destruction of the cells adjacent to the arterioles and venules (Plate No. 32). The cytoplasm was heavily loaded with granular deposition which marked the nuclear and cytoplasmic differentiation. Another characteristic feature of the 28 days treated liver was that it showed the appearance of neoplastic cells inside the arterioles and venules (Plate No. 32).

Continuation of the administration of the azo-dye-azocarmine for a period of 35 days showed the appearance of a huge quantity of cytoplasmic granules. Vacuolar structures appeared and the blood vessels were heavily invaded by the neoplastic cells (Plate No. 33). The size of the nucleus was found to be markedly bigger than that of the control.

The histological study of the 42 days treated liver revealed that the neoplastic cells invaded through the walls of the blood vessels and as a result the latter disintegrated (Plate No. 34). The size of the nucleus was found to increase. Invasion of the liver tissues from the cells occurring inside the blood vessels
**PLATE No. 36**: Microphotograph showing the dissolution of some of the nearby cells of the blood vessels and the invasion of the liver cells by the transformed cells.

**PLATE No. 37**: Microphotograph showing the destruction of arterioles and venules after 56 days of carcinogen treatment.
was prominent (Plate No. 35).

Continuation of the invasion of the liver cells by the transformed cells from the blood vessels and dissolution of some of the nearby cells was marked (Plate No. 36) after 49 days of carcinogen treatment. Vacuolar structures were apparent along with the increase of granular substances. Loss of normal hexagonal cell structure with loss of normal cellular association was marked. The lobular pattern of the liver histology was more or less completely lost due to the loss of cementing materials between the hepatic cells.

Liver cells were marked by heavy deposition of cytoplasmic granules after 56 days of treatment. Nuclei increased in size. Vacuolar spaces were found. The most prominent feature of 56 days treated material was that the arterioles and venuoles were destroyed (Plate No. 37) along with cellular cirrhosis. Nuclear and cytoplasmic distinction was very meagre. The loss of histopattern was comparable to that of the 49 days treated guinea-pigs.

The structure and appearance of lesion was observed after 7 days of treatment which revealed the formation of nodules with enlarged cells encaptulated in fibrous covering and clearly demarcated from the rest of the liver tissue. Later, the advancement of such cancerous growth had been traced through successive stages and the drastic changes in the breakdown of the arterioles and venuoles along with the invading behaviour were noticed.
3. **REVIVAL EXPERIMENT (VITAMIN TREATED SET)**

Guinea-pigs after the experimental induction of carcinogenesis in the liver was treated with vitamin C in order to study the role of vitamin C in interfering with the carcinogenic changes in the cellular level. The continuation of treatment for a period of about 21 days after the carcinogenesis was established with the hepatocarcinogen-azocarmine, a marked recovery effect was noticed in the histological picture of the liver which indicates a definite revival trend induced by the vitamin C treatment in case of induced chemical carcinogenesis. The administration of considerable amount of vitamin C gradually produced an overall revival effect which appeared to be very near to that of the control liver structure after a treatment for about 56 days. The number of lesion and tumour was also reduced considerably in the vitamin C treated set.

The studies on the histology of the liver treated with vitamin C after the induction of carcinogenesis as described below indicated the regenerative, healing and anticarcinogenic effects of vitamin C in cellular level.

The administration of vitamin C to the carcinogen treated set of guinea-pigs reacted with the carcinomatous changes of the liver and overall revival effect was observed. The partially damaged liver tissues responded the vitamin which gradually induced certain recovery effects probably due to the healing and antitumour properties of vitamin C.

The induction of carcinogenic changes was revealed by gradual loss of cementing material between the hepatic cells.
PLATE No. 38: Microphotograph showing the revival effect of vitamin C on the carcinogenic changes induced by the hepatocarcinogen-azocarmine after 28 days of treatment.

PLATE No. 39: Microphotograph showing the revival effect of vitamin C on the carcinogenic changes induced by the hepatocarcinogen-azocarmine after 56 days of treatment.
The compact nature of the hepatic cells was not observed in.

After 14 days of treatment the liver exhibited a gradual appearance of cellular adhesiveness. Nuclear size was found to be enlarged. The distinction of the nucleus and the cytoplasm was markedly increased after 14 days of vitamin C administration, which was visibly lost with carcinogenic induction. The nucleus was distinct.

Continuation of treatment upto 21 days revealed the appearance of some granular structures in the cytoplasm of the treated liver cells. There was a marked increase in the cementing material between the cells with continuation of vitamin C administration. The nucleus was found to be slightly enlarged in size with increased basophilic reaction.

Continued administration of vitamin C had a revival effect both in the formation of cementing material as well as the adjacent cells of the blood vessels (Plate No. 38) in 28th day treated liver cells. Histological picture did not exhibit the dissolution of the adjacent cells of the blood vessels as was seen in the carcinogen treated set of the experiment. It also revealed the presence of some neoplastic cells inside the blood vessels (Plate No. 38) and some vacuolar structures in the liver cells but the general cell structure was partially revived with accumulation of cementing material between the cells.

After 35 days of treatment with vitamin C the liver cells showed a gradual loss of the granular particles in the
cytoplasm which was seen in the carcinogen treated set of the experiment. A gradual uniformity in the cytoplasmic structure was observed. A marked change in the nature of the cells from the carcinonomatous to normal hepatic cell pattern was observed.

Continuation of the treatment with vitamin C had a revival effect on the nuclear size after 42 days of treatment. Presence of the malignant cells inside the blood vessels were still observed. However, a revival in the nature of cellular adhesion with transformation to more or less normal hepatic cell form was marked.

Treatment with vitamin C by 49th day of the revival experiment showed a gradual tendency of the liver cells to revive to its normal counterpart. The cirrhotic condition of the second set of experiment was lacking. The cellular adhesiveness reappeared with the regaining of the normal histological structure of the liver except the presence of few vacuolar spaces.

The later part of the experiment showed an overall trend of the hepatic cells to return to its normal state after the administration of vitamin C. The nuclear size was restored, cellular cytoplasm was comparable to that of the control and the vacuoles gradually disappeared (Plate No. 39).

Histological studies revealed that the malignant cells which appeared in the arterioles and venules completely disappeared which definitely indicated a marked inhibition in the process of carcinogenic transformation of liver cells, in response to prolonged vitamin C treatment. Which may be further ascertained from the reappearance of the cementing material
between the cells and the regaining of cellular adhesiveness, which was totally lost with progressive advancement of induced carcinogenesis.

4. Discussion:

Findings of the present experiment therefore reveals successive histological changes in liver cell during azocarmine induced neoplastic transformation and subsequent revival effects after prolonged administration of vitamin C. Cellular proliferation is a constant feature of neoplasia. This cellular proliferation leads into the hyperplastic growth. Berenblum (1970) postulated that the neoplastic cells did not have the growth equilibrium, and this loss of equilibrium results from a delay in maturation of the neoplastic cells. He further stated that a malignant tumour growing in a cellular organ of fairly uniform consistency (e.g., liver) tend to invade in all directions, though microscopically the growing edge was far from regular. Under these conditions even the thin outer capsule of the organ acts temporarily as a barrier, the tumour finding it easier to invade the other cellular tissue than the capsule. Another example of this principle of invasion along the lines of least resistance is that of lymphatic permeation. Within the lumen of a lymphatic channel, after the tumour cells have gained entrance through the wall of the vessel as revealed by the experimental findings in the arterioles and venules and then subsequent invasion in the organ. In such a case the growth may extend rapidly and reach very far from the primary site of entry. Penetration through lymphatic wall or for that matter
through walls of arterioles and venules, and even large arteries and veins were observed. Berenblum (1970) reported a variability in structure not only as between one tumour and another derived from the same cell type of origin, but also in different parts of the same tumour, constitutes yet another architectural derangement in malignancy. The more malignant the tumour, the more marked are the variations. Malignant cells tend to vary in size, and in the shape and size of their nuclei.

The study of cells by electron microscopy has brought to light a wealth of details not visible by the light microscope. The connective tissue stroma of a malignant tumour may be deficient as though the growth of the tumour is too rapid for the stroma reaction to keep pace with it. The accompanying deficiency in the blood supply may lead to central necrosis of the tumour or the formation of immature blood vessels, which may appear as mere vascular clefts among the tumour cells, with hardly any endothelial lining.

Metastasis is a secondary centre of tumour growth at a distance from the primary focus. It is derived from transported live cells of the primary mass, which it resembles both in histological structure and in functional behaviour. Minor differences between the primary tumour and its metastases may result from local modifying factors. Regarding the way metastases are formed the simplest and most plausible concept first to be considered was that they arose by embolic dissemination of detached tumour fragment set free in the blood stream either directly or penetration of blood vessels. Since capillaries and
vessels are more easily invaded by tumour cells than arterioles, it was to be expected that dissemination peripheral to the local would be rare. In the present experiment the presence of neoplastic cells inside the blood vessels definitely disclosed the metastatic nature of azocarmine induced carcinogenesis.

Bannasch and Muller (1964) reported that the changes in the cytoplasm of liver cells during the precancerous phase at the centre of the lobules were basically different from those at the periphery. To describe these phenomena as "acinuscentral" and "aminusperipheral" in accordance with Kiernan's (1833) strictly morphological classic concept of liver lobules is, however, not fully satisfactory. At least during the first weeks of the experiment the histotoxin pattern shows a definite correlation with the different zones of vascularization described by Rappaport, et al. (1954) which are collectively attributed to a "functional" liver acinus. But the concept of Rappaport suffers in that a cirrhotic reorganization of the liver parenchyma occurs in the later stages of the experiment, leading to a considerable disturbance of normal blood flow.

Wallach (1968) reported that malignant transformation may change the cell surface in a way that abolishes growth control. Loss of cellular adhesiveness with subsequent loss of hexagonal outer surface with progress of carcinogenic changes was also observed in the present experiment. He pointed out that mutations altering the structure of plasma membrane may affect nuclear, mitochondrial and endoplasmic membrane as well, and lead to an array of metabolic, structural and behavioural
alterations. These findings support the postulate that cancer might be, in the last analysis, a membrane disease. On the other hand, electron microscopic studies by Porter and Bruni (1959) revealed that in animals fed dimethyl-aminoazobenzene, glycogen disappeared whereas the smooth membrane of the endoplasmic reticulum hypertrophied.

As regards liver, Miller and Miller (1947) showed that the ingestion of azo-dyes led to the formation of protein bound dye which appeared very rapidly after the carcinogen was given. At first the amount of this complex increase with the time of ingestion and then decreases, to disappear completely from the tumour while the apparently healthy tissues surrounding the nodule still contain it. It was showed by Hultin (1957) that the protein dye complex appeared first in the microsomal fraction, after a certain time of protein bound dye was present in all the cell structures but more than half of it was found in the soluble cytoplasmic phase. These results have been confirmed by Gelboin, et al. (1958), who added that the dye was bound to certain liver protein during their synthesis rather than to proteins. Saito (1973) studied the cytologic manifestations of lymphomatoid granulomatosis in cerebrospinal fluid and found predominantly undifferentiated medium sized cells, which were retrospectively interpreted as reticular cells. These cells were characterized by an atypical nucleus with one or more prominent nucleoli or clumped chromatin granules, abundant cytoplasm and frequent amoeboid protrusions of the cytoplasmic membrane. Small numbers of undifferentiated plasmacytoid cells, lymphocytes, neutrophils, and erythrocytes were evident. Savelev
and Tseshkovskii (1975) reported the presence of vacuoles in the nucleus and cytoplasm of malignant lymphoid cells.

The histological criteria of malignancy on which most reliance is normally placed are — (a) evidence of invasion, and (b) disturbance in the organization of the tissue. In the present experiment both had been marked. Invasion through the blood capillaries was found to be a common experience in the second set of the experiment along with a modification in the structure of the cells. The application of vitamin C to the cancer induced guinea-pigs showed the normal histological structure of the liver. But the mechanism how the carcinogen acts and the vitamin C acts needs further studies on enzymatic level.