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Ultrastructure and movement of the ependymal and tracheal cilia in congenitally hydrocephalic WIC-Hyd rats

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Abstract. The aim of the present investigation is to determine whether or not hydrocephalus occurring in hydrocephalic Wistar-Imamichi strain rats (WIC-Hyd) is caused by functional and structural disorders of ependymal cilia. Ultrastructures and movement of cilia in the ependyma of the lateral, III and IV ventricles and aqueduct of Sylvius and in the trachea walls of the animals were examined by means of scanning electron microscopy (SEM), transmission electron microscopy (TEM), and light microscopy using a phase-contrast microscope equipped with a high-speed video recording system. SEM revealed that a marked decrease in the length and number of cilia in the ependymal and tracheal walls occurred in affected male WIC-Hyd. This finding was noted even before the development of ventricular dilatation and was not related to the degree of ventricular enlargement after development of hydrocephalus. A moderate decrease in length and number of cilia was also seen among the normal ciliary tufts in affected female rats which developed a mild degree of hydrocephalus. TEM cilia findings included abnormal axonemal structures such as a lack of dynein arms and displacement of microtubules. The incidence of these ultrastructural abnormalities was found to be greater in affected male rats than in affected female rats. All cilia in affected male rats before and after development of hydrocephalus were immotile. A variety of movement disorders such as immobile, rotatory, and vibratory cilia were observed beside normally beating cilia (motile cilia) in affected female rats which never developed hydrocephalus as severe as that seen in affected male rats. These results seem to indicate that there is a correlation between cilia movement disorder and the degree of ultrastructural abnormalities. Consequently, hydrocephalus developing in affected male and female WIC-Hyd appears to be caused by a motility disorder of ependymal cilia which is part of the primary ciliary dyskinesia (PCD) affecting these animals. The present study appears to indicate that the movement of ependymal cilia may play a role in cerebrospinal fluid circulation, and that dysfunction of ependymal ciliary movement may contribute to development of hydrocephalus in WIC-Hyd rats.

Key words: Ependymal cilia – Congenitally hydrocephalic rat – Situs inversus viscerum – Kartagener syndrome – Immotile cilia syndrome – Primary ciliary dyskinesia

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We have reported [25, 26] that WIC-Hyd rats found in the Wistar-Imamichi strain rat breeding colony at the Research and Development Division of Chugai Pharmaceutical Co., Ltd., Tokyo, are affected by a high incidence of congenital hydrocephalus. A difference between males and females in the severity of hydrocephalus was noted, that is, approximetely 34.9% of the females developed slowly progressive and/or arrested hydrocephalus, although enlargement of the head was not conspicuous. Regardless of this disease condition, they grew up to maturity and became capable of reproduction, and they seldom died of hydrocephalus. On the other hand, approximately 33.7% of the males were affected by rapidly progressive hydrocephalus where there was manifestation of ventricular dilatation resulting in dome-shaped enlargement of the head as early as 1 week after birth, and all of the animals died of raised intracranial pressure (ICP) due to triventricular hydrocephalus caused by secondary stenosis of the aqueduct of Sylvius within 1 month after birth. We suggested that breeding data indicated hydrocephalus in WIC-Hyd rats was communicating, heritable, and X-linked [25]. Also noteworthy was that approximately one-half of the males with hydrocephalus were found to have total situs inversus viscerum, while neither female rats with nor those without hydrocephalus ever developed such a visceral abnormality. Since situs inversus viscerum is known to be closely related to Kartagener

syndrome (KS), we speculated that WIC-Hyd rats might be KS animals.

Regarding KS in human, in 1903 Siewert [44] reported a case complicated by bronchiectasis and situs inversus viscerum. During the period 1933–35, Kartagener [21, 22] demonstrated that bronchiectasis, situs inversus viscerum, and chronic sinusitis were frequently mutually complicated. This disease condition came to be known as Kartagener syndrome. In 1977, Eliasson et al. [14] reported a series of cases in which the syndrome was complicated by male sterility and chronic respiratory infection and proposed that the syndrome might be caused by a lack of or reduction in flagellar and ciliary motility. They called this disease condition "immotile cilia syndrome" (ICS) [14]. In 1981, Sleigh et al. [47] proposed the designation "primary ciliary dyskinesia" (PCD), and then KS and ICS became known as subgroups of PCD because not only ciliary immotility but also various types of ciliary dysfunction [35, 38, 40, 52] were found in bronchial cilia and flagella in KS and ICS. PCD is now considered a systemic ciliary dyskinetic disease. On the basis of the facts, we also speculated that WIC-Hyd rats might have ciliary diskinesia in the trachea and the cerebral ventricle, and would serve as a good animal model for specific PCD hereditary diseases.

In 1943, Olsen [32] described 11 out of 85 patients with dextrocardia (but not KS) to have other defects that included congenital heart disease, hydrocephalus, imperforate anus, cleft palate, and others. To date, six patients with hydrocephalus in association with PCD have been reported [4, 13, 18, 20], but no one has proved an etiological correlation between induction of hydrocephalus and PCD of ependymal cells. In the present study we investigated whether or not WIC-Hyd rats are affected by ciliary dyskinesia in terms of the pathogenesis of hydrocephalus. This was accomplished by means of scanning and transmission electron microscopy (SEM and TEM) and ciliary observation under a phase-contrast microscope utilizing a high-speed videographic technique.

Materials and methods

WIC-Hyd rats were used: 14 normal male rats without hydrocephalus aged from 3 days to 1 month; 11 normal female rats without hydrocephalus aged from 3 to 12 months; 12 female rats with mild hydrocephalus aged from 3 to 12 months; and 19 male rats, aged from 3 days to 1 month with severe hydrocephalus and/or normal ventricular size associated with situs inversus viscerum, the latter category of which it was presumed would develop hydrocephalus if allowed to survive.

Scanning electron microscopy of ependymal and tracheal cilia

Brains and tracheae isolated, respectively, from 6 males and 6 females without hydrocephalus, 7 females with mild hydrocephalus, and 8 males with severe hydrocephalus with or without ventricular dilatation but with situs inversus viscerum, were fixed in 2.5% glutaraldehyde solution for 2 h. Tissue slabs dissected from anatomically designated portions, including the lateral and medial walls of the frontal horn of the lateral ventricle, the walls of the III ventricle, the aqueduct of Sylvius, the floor and roof of the IV ventricle, and the anterior and posterior walls of the middle portion of the trachea, were then washed in cold 0.15 *M* phosphate buffer and postfixed in 2% osmium tetroxide. Because of known regional differences in ependymal configuration, the sample materials studied as described above were taken from similar locations in all instances. After osmium fixation, all specimens were again washed in cold 0.15 *M* phosphate buffer and dehydrated in ethanol, critical point-dried with amyl acetate, coated with gold-palladium, and viewed with a Jeol JSM-800 SEM.

Transmission electron microscopy of ependymal and tracheal cilia

For TEM, samples from each ventricular and tracheal wall adjacent to those used for SEM specimens were prepared. The tissue slabs were fixed, postfixed, and dehydrated in the same fashion as SEM specimens, and were then treated with propylene oxide and embedded in epoxy resin. Ultrathin sections were prepared with an LKB 8800 microtome, stained with lead citrate and uranyl acetate, and examined with a Jeol JEM-100C TEM.

Phase-contrast microscopy of movement of ependymal and tracheal cilia

The animals used for this investigation were 11 males, including those with severe hydrocephalus and others with normal ventricular size but having situs inversus viscerum, 8 males without hydrocephalus, and 5 females with or without mild hydrocephalus. The brain and trachea were removed immediately after decapitation and immersed in Hank's solution at room temperature. The upper and inner walls of the frontal horn of the lateral ventricle, the lateral wall of the III ventricle, the aqueduct of Sylvius, the floor and roof of the IV ventricle, and the anterior and posterior walls of the middle portion of the trachea were exposed under an Olympus dissecting microscope with sufficient care to avoid touching ciliated surfaces with instruments. Small pieces of the ependyma and epithelium were excised with very sharp curved corneal scissors held parallel to the ependymal or epithelial surface in order to obtain a specimen with a thin edge. The ependymal wall surfaces of these specimens were placed tangential to the base of a Petri dish filled with Hank's solution. Movements of the cilia were examined by means of a Nikon phase contrast microscope at $\times 400$ magnification and were recorded on VHS videotape with a Nac MHS-200 high-speed videogram. The frequency and mode of the ciliary beats were assessed by analyzing the recorded videotapes. The mean time lapse between removal of the examination materials and initiation of recording was about 10 min. Ciliary motility can proceed for as long as 3 h after removal of specimens from an animal, but observation was carried out for an average period of 1 h.

Results

Scanning electron microscopy of ependymal and tracheal cilia

In unaffected male and female rats without hydrocephalus (Fig. 1 A), the ependymal surface on the frontal horn of the lateral ventricle was covered with regularly spaced tufts of cilia. Each of the tufts consisted of 20–40 cilia, each 4–6 μm in length and 0.2–0.3 μm in diameter, and fixed in the same direction (Fig. 2 A). Cilia on the walls of the III and IV ventricles and the aqueduct of Sylvius were similarly fixed in one direction. Tracheal

cilia existed among goblet cells, forming thick clusters. In the affected female rats with mild hydrocephalus (Fig. 1 B), the most prominent changes were observed in the ependymal and tracheal cilia. They were shortened and fewer in number, and directional abnormalities, with some cilia extending to full length and other cilia forming clumped clusters, were observed among the normal ciliated tufts (Fig. 2 B). In all samples, the greater part of normal cilia were replaced by abnormal cilia, and these abnormalities were more prominent in the affected male rats with situs inversus viscerum which had not yet developed ventricular dilatation (Fig. 2 C). Identical findings after the development of ventricular dilatation (Fig. 1 C), and similar abnormalities in the III and IV ventricles, the aqueduct of Sylvius, and the trachea also were observed.

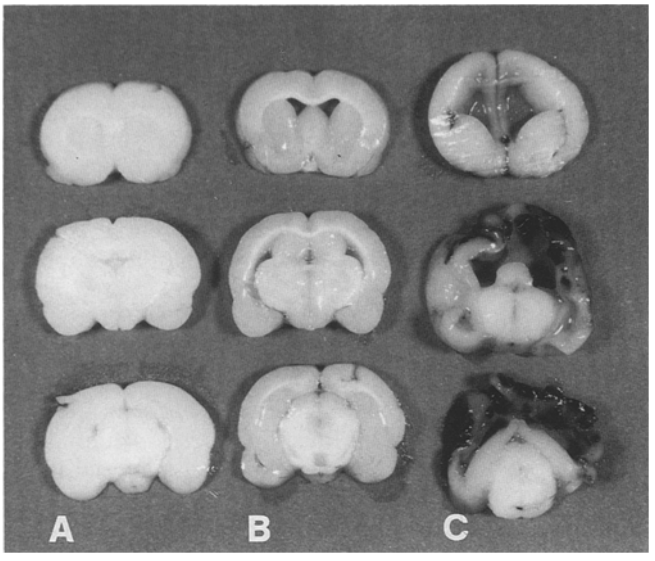


Fig. 1 A–C. Coronal rat brain sections at the anterior horn (*upper*), body (*middle*), and posterior horn (*lower*) levels of the lateral ventricle of (**A**) a 24-day-old unaffected male rat and (**B**) a 12-month-old affected female rat with mild ventricular dilatation. **C** Similar sections of a 24-day-old affected male rat with hydrocephalus show marked ventricular dilatation with intraventricular hemorrhage and subdural hematoma

Transmission electron microscopy of ependymal and tracheal cilia

Shown at left in Fig. 3 is a cross-section of ependymal cilia on the lateral wall of the frontal horn in the lateral ventricle of a 3-day-old normal male rat without hydrocephalus where no axonemal abnormality was found in any structures, including inner and outer dynein arms, spokes, and microtubular alignment. In contrast, an axoneme of the ependymal cilia in a similar region in a 3-day-old affected male rat with total situs inversus viscerum, which had not yet developed ventricular dilatation, was found already to be abnormal, as shown at right in Fig. 3, indicating such various structural abnormalities as the absences of (1) inner and outer dynein arms, (2) peripheral linkages, and (3) central complex, and abnormal microtubular alignment. Similar findings were apparent in affected male rats already hydrocephalic. These findings also were noted in the III and IV ventricles, the aqueduct of Sylvius, and the trachea. Such abnormal cilia also were observed in affected female rats with mild hydrocephalus, but the incidence of ciliary structure abnor-

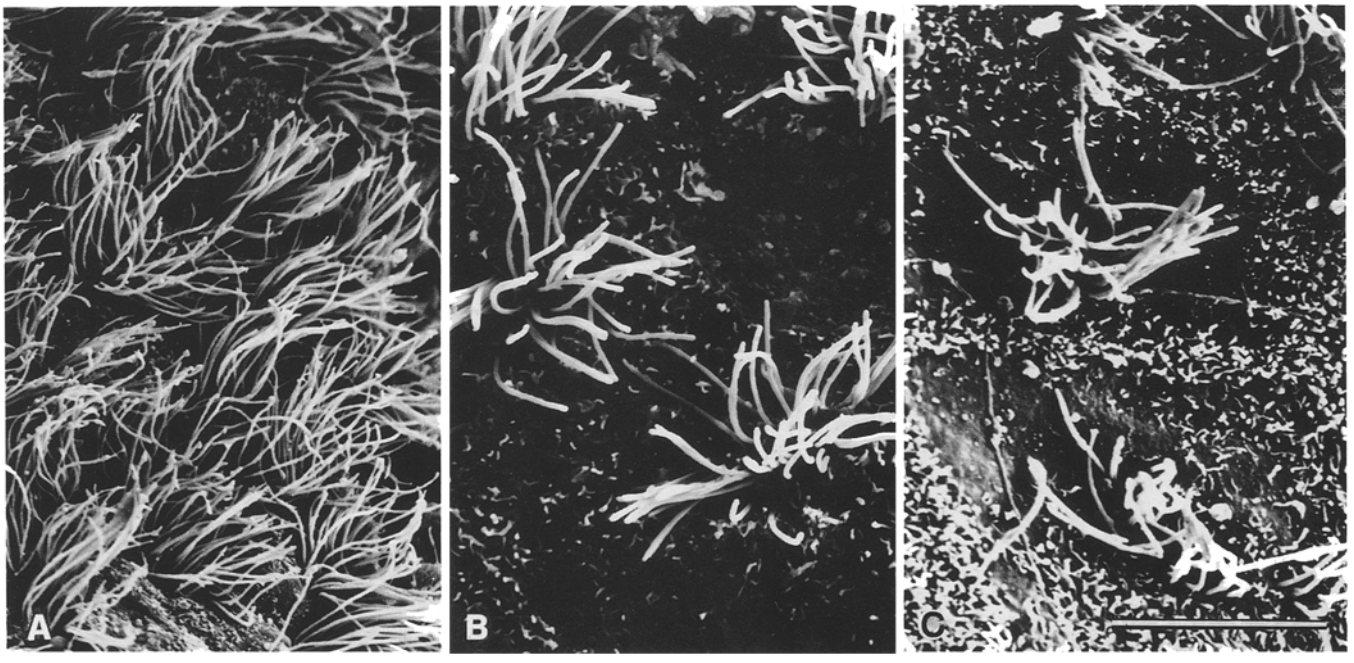


Fig. 2 A–C. Scanning electron micrographs of ciliary processes of upper ependymal wall of lateral ventricle frontal horn depict (**A**) normal ciliary appearance in a 3-day-old unaffected male rat, (**B**) shortened and decreased cilia in a 3-month-old affected female rat with mild hydrocephalic dilatation, and (**C**) markedly decreased and randomly oriented cilia in a 3-day-old affected male rat with situs inversus viscerum which has not yet developed hydrocephalus. *Bar* = 10 μm

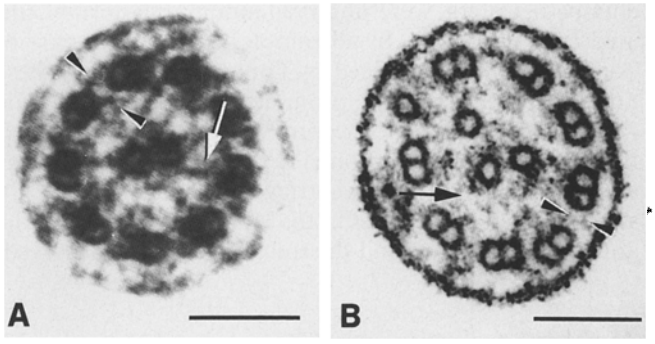


Fig. 3A, B. Transmission electron micrographs of horizontal cross-sections of cilia, depicting (**A**) normal axonemal structures such as inner and outer dynein arms (*arrowheads*) and radial spokes (*arrow*) in a 3-day-old unaffected male rat and (**B**) the lack of both dynein arms (*arrowheads*), displacement of the peripheral tubules and the lack of radial spokes (*arrow*) in a 3-day-old affected male rat with situs inversus viscerum which has not yet developed hydrocephalus. *Bar* = 0.1 μm

malities was less than in the case of affected males with/without hydrocephalus.

Phase-contrast microscopy of movement of ependymal and tracheal cilia

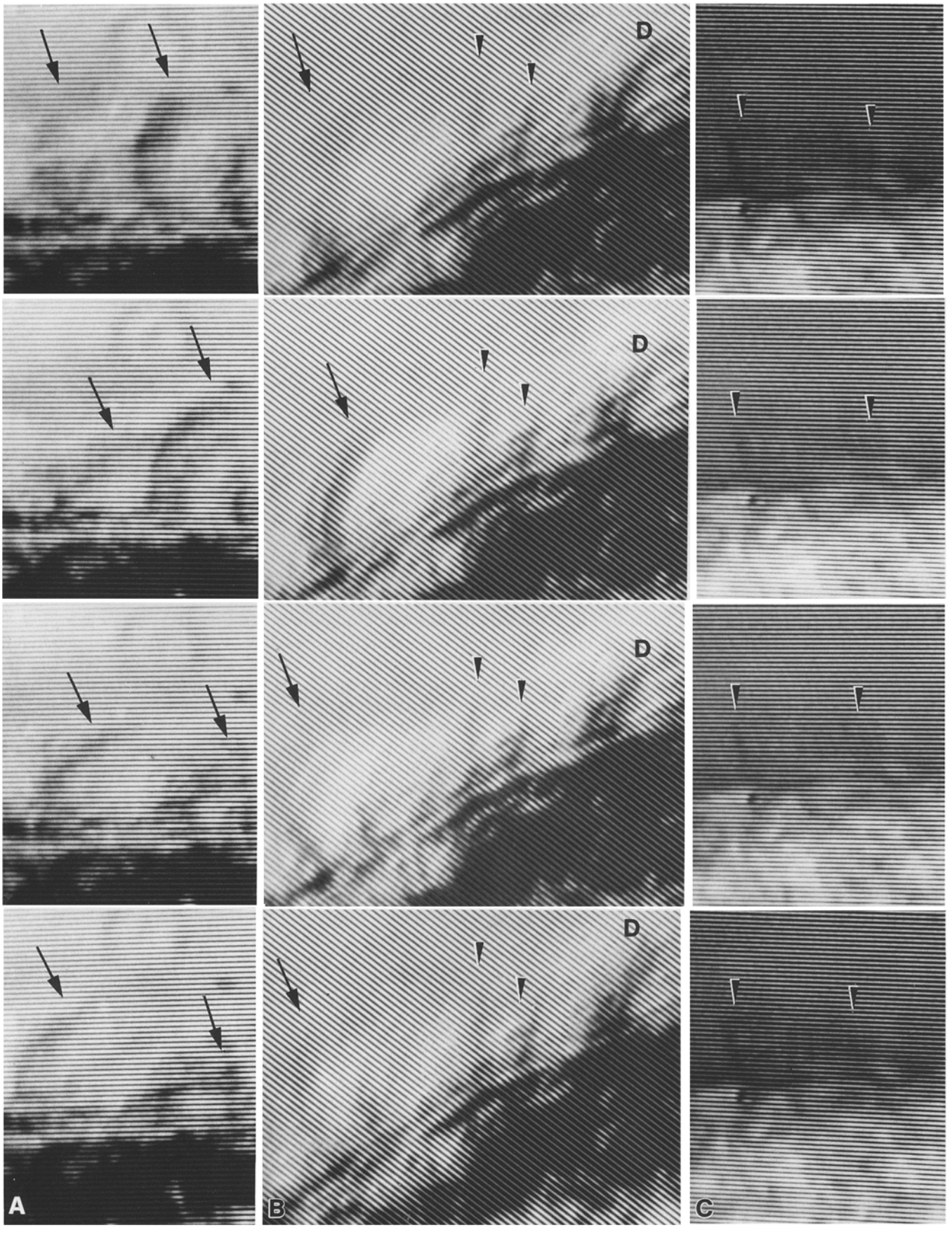
High-speed-videotaped static images of the movement of ependymal cilia on the frontal horn of lateral ventricle are shown in Fig. 4. Each cilium in an unaffected male rat without hydrocephalus (Fig. 4A) beat with a rapid and uniform motion. In other unaffected male and female rats without hydrocephalus, cilia of the lateral, III and IV ventricles, the aqueduct of Sylvius, and the trachea showed a metachronal movement and beat with a frequency of 1000–2000 cycles per minute. In the hydrocephalic female rat (Fig. 4B), the ciliary movement was characterized by various patterns, including normal to-and-fro movement, vibratory and rotatory motion, and akinesia; that is, the synchronized to-and-fro movement of the cilia in a certain direction observed in unaffected rats without hydrocephalus was completely perturbed by the mosaic distribution of ciliary tufts and different modes of movement, and the number of cells with motile cilia was lower than in normal subjects. Similar findings were observed on the walls of the III and IV ventricles, the aqueduct of Sylvius, and the trachea in other affected female rats with hydrocephalus. Affected male rats with situs inversus viscerum which had not yet developed ventricular dilatation already demonstrated immotile cilia (Fig. 4C). In other hydrocephalic males, none of the ependymal and tracheal cilia were found to show any beat, regardless of ventricular size.

Discussion

The results obtained in the present study demonstrated that affected male WIC-Hyd rats with situs inversus viscerum, before and after development of ventricular dilatation, have immotile cilia in the ventricular ependyma and trachea, leading to the assumption that abnormality in the axonemal structure of cilia might have a role in the pathogenesis of ciliary motility disorder. Contrarily, affected female WIC-Hyd rats with mild hydrocephalus were found to have a lesser degree of axonemal structural abnormality and to display a mosaic mode in ciliary movement due to the irregular distribution of kinetic, dyskinetic, and immotile cilia both in ventricular ependyma and trachea. From these observations, one could describe affected male and female rats respectively as modeling human ICS and PCD. There also was a strong suggestion that abnormal axonemal structures of the cilia, such as the absences of dynein arms, radial spokes, and microtubules, might have a role in the pathogenesis of ciliary motility disorder. A potential correlation between morphological changes of the axonemal structure and the functional alterations of ciliary movement in WIC-Hyd rats, as described below, will be discussed.

Although there have been many reports in regard to SEM images of ependymal cilia in experimental hydrocephalus induced by various methods, including intraperitoneal viral infection, intrathecal bacterial infection, or intracisternal injection of kaolin or silicone oil [17, 31, 33, 55], only a few reports have been published in regard to SEM investigation of ventricular cilia in animals with congenital hydrocephalus [9, 23]. In the assessment of SEM images of the lateral ventricular wall in rats with hereditary hydrocephalus, Lindberg et al. [27] noted that the cilia were shortened, fewer in number, and clumped or matted. Using SEM, Bannister and Mundy studied ependymal surfaces of the ventricles of two congenitally hydrocephalic Hy-3 mice in comparison with that of an infant with hydrocephalus complicated by meningocele and Chiari malformation. In the basal regions of the lateral ventricles of the Hy-3 mice, the ependymal wall possessed normal cilia, but the upper ventricular wall was found to be devoid of cilia, whereas no abnormal change in ependymal cilia in the lateral ventricle of the infant was noted [7]. They concluded that the changes in the ventricular wall and the coarse ependymal cilia could be attributed to thinning of the ventricular wall resulting from increased ICP due to hydrocephalus, and were not the cause but the result of hydrocephalus. By contrast, Afzelius [4] described the defective cilia as being the primary cause of hydrocephalus and the high intraventricular pressure as its consequence. In the affected male rats with situs inversus viscerum in this study, the changes in ependymal surfaces, where the ependymal

Fig. 4A–C. Four frames of a videogram show (*top* to *bottom*) successive changes at 0.005-s intervals in (**A**) a 3-day-old unaffected male rat (*arrows* in each frame indicate ciliary beat), (**B**) various kinds of cilia such as immotile (*arrowheads*), dyskinetic (*D*) and beating cilia (*arrows*) in a 3-month-old affected female rat with mild hydrocephalus, and (**C**) ependymal cilia, all immotile (*arrowheads*) in a 3-day-old affected male rat with situs inversus viscerum which has not yet developed hydrocephalus. Magnification × 8000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cilia were coarse, shortened, and with thin diameters, and/or clustered or entangled in multiple directions, were found prior to ventricular enlargement. These findings would suggest that morphological changes of the cilia are not due to increased intraventricular pressure or stretching of the ependymal walls secondary to ventricular enlargement, but rather than they are a potential cause of hydrocephalus.

The relationship between axonemal structure and the movement of cilia and flagella has been studied in investigations using cytozoons, sea urchins, and tetrahymenae [2, 16, 19, 29, 51]. Many reports have been published in regard to correlation between the ultrastructure and the motility of the tracheal cilia and the spermal flagella in human cases [3, 4, 14, 35, 36, 47]. The first step in unraveling ICS was the discovery by Pedersen and Rebbe [36] of a case of immotile spermatozoa devoid of the dynein arms. Afzelius studied four subjects who produced immotile sperm, three of whom had frequent episodes of bronchitis and sinusitis where there was no mucociliary transport. He found that cilia from cells of these patients lacked dynein arms [3]. These studies strongly suggested that the absence of dynein arms was the cause of immotility of spermatozoa and cilia. In addition to the complete absence of both dynein arms, defects of the inner or outer dynein arms, spoke heads, one or both microtubules, and the central sheath also have been implicated as axonemal abnormalities of sperm and cilia in cases of motility disorder [5, 49, 50, 54]. Following these observations, the mechanism of ciliary or flagellar beats was investigated by various scientists and found to be based on the sliding and/or switching of doublet tubules by dynein arms (ATPase) [15, 42, 43], and it also was shown that the cyclic-AMP-dependent proteinase and the regulatory proteins constituting ciliary substance intermediately work to create the coordinate and metachronal movement of cilia and flagella [41, 45, 46].

With regard to hydrocephalus and ependymal ciliary dyskinesia, Bryan studied mice homozygous for a recessive, pleiotropic, mutation hydrocephalic-polydactyly (hpy) and found that the male hpy mouse was affected by postnatal hydrocephalus and complete sterility and that the female hpy had reduced reproductive performance. He suggested that the fertility problems and the development of hydrocephalus could have arisen as consequences of defective flagella and ciliary axonemes of oviduct and ependyma [10].

A small number of patients in whom hydrocephalus was associated with PCD have been reported to date [4, 13, 18, 20, 32]. The reason for the small number reported is probably due in part to the low incidence of PCD, to the lack of recognition of the possibility that PCD may be associated with mild hydrocephalus, and to insufficient medical examination. Following his assessment in 1943 of 85 patients with congenital dextrocardia, Olsen [32] mentioned that 11 of them had other defects including hydrocephalus, and that 16.5% (14/85) of them had bronchiectasis. Afzelius [4] examined the brains of seven patients with ICS by CT. In three of these patients, the ventricular system and sulci were slightly enlarged. Greenstone et al. [18] reported a 12-year-old boy with PCD and bronchiectasis who had developed hydrocephalus in the neonatal period. They mentioned that the concurrence of hydrocephalus and PCD was of interest, because it might suggest a function of cilia in the embryonic development of CSF pathways. Jabourian et al. [20] observed a 15-year-old girl with KS, an immotile ciliary disorder consisting of sinusitis, bronchiectasis, and situs inversus viscerum, who developed persistent headaches and was found to have communicating hydrocephalus with evidence of impairment of CSF circulation at the level of the tentorium. This patient required a ventriculoperitoneal shunt, after which there was relief of the hydrocephalus and its associated symptoms. These authors assumed that ependymal cilia normally kept the aqueduct patent and that the risk of stenosis was increased in patients with ciliary immotility. De Santi et al. [13] reported a particular form of PCD (ciliary aplasia) in a girl with bronchiectasis who developed hydrocephalus in the neonatal period. CT showed triventricular hydrocephalus caused by aqueductal stenosis, then a ventriculoatrial shunt was inserted and her subsequent neurological development was normal. De Santi et al. suggested that ependymal ciliary defects in PCD might be a contributory embryological factor in the pathogenesis of particular forms of hydrocephalus, and that there might be a causal relation between PCD and hydrocephalus. These previous investigations by various scientists lend support to our speculation that abnormal fine structures of ependymal and tracheal cilia may be intimately related to ciliary motility disorder and hydrocephalus.

Purkinje [37] was the first scientist to observe the movement of cilia on the walls of cerebral ventricles in full-term sheep fetuses, and he found that the cilia were long and pointed and vibrated in a whip-like fashion. To date, there have been several reports on the relation between the beat of cilia and CSF currents on the ventricular surface [1, 11, 12, 30, 39, 48, 53, 56, 57]. Konno and Shiotani [24], utilizing a phase-contrast microscope, reported that the red blood corpuscles moved toward the foramen of Monro in the lateral ventricle of 2- to 8-week-old dogs. Worthington and Cathcart [11, 56, 57] observed the beating cilia in surviving ependyma of adult human brains and then mapped the ciliary currents in each of the ventricles. They concluded that the function of cilia in the central nervous system is to maintain the CSF in constant motion and to clear cellular debris from the ependymal surface of the ventricular system. Milhorat [28] proposed that the strong cilia-induced currents near the ventricular walls are important for intraventricular CSF circulation, but others [6, 39, 52] attributed only a minor role to the cilia, and some [34] regard them as nonfunctional rudimentary structures. Utilizing SEM, Yamadori and Nara [58, 59] observed in adult mice the directions of ciliary beat on the walls of the whole brain ventricular system, including the central canal, and revealed that the pattern of directions of the ciliary beat was always the same in each individual in the series despite different positions at the time of fixation, and the pattern was in agreement with the anticipated flow of the fluid. Yamadori then concluded that the ciliary movement has a close relationship with the CSF current and must be an important

factor in producing and regulating the flow of CSF within the ventricular system.

The facts established by the aforementioned investigators allow us to hypothesize that the flow of the CSF might be variously restricted by impairment of ciliary movement, especially at the aqueduct of Sylvius, which is an extremely narrow canal for the passage of CSF, leading to the development of hydrocephalus. Hence, marked CSF stagnation due to ICS might produce high intraventricular pressure and generate enlargement of the lateral and III ventricles in hydrocephalic males, while slight CSF stagnation owing to ciliary dyskinesia might result in mild ventricular dilatation in hydrocephalic females. Although the pressure gradient between CSF formation and absorption, the pressure variations associated with arterial pulse and respiration, and the continuous outpouring of new CSF are thought to be important factors when considering the hydrodynamics of the ventricular system [8, 28, 39], our findings clearly show that the movement of ependymal cilia may also play an important role in CSF flow and that the dysfunction of ependymal ciliary movement may contribute to the ventricular dilatation associated with hydrocephalus in cases of PCD.

Noteworthy in the present experimental model are the findings that hydrocephalic males demonstrated progressive hydrocephalus which might belong in the ICS category, and that hydrocephalic females demonstrated slowly progressive and/or arrested hydrocephalus which might pertain to the PCD genre. It is believed the present model could well serve as a good way of studying the pathogenesis of PCD. Further studies on the distribution, activity, and function of human and animal ependymal cilia, as well as the relations between ciliary motility and CSF dynamics, also are warranted.

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