THE ROLE OF SEX AND CHROMOSOMAL ABNORMALITIES IN THE DEVELOPMENT OF MURINE EMBRYO-DERIVED TUMORS

R. J. VAN BERLO

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STELLINGEN

I. Tumoren zijn karikaturen van het proces van weefselvernieuwing.

II. Gendosis effecten spelen een belangrijke rol in de pathogenese van, door embryotransplantatie geïnduceerde, tumoren in de muis.

III. De gevonden polyploïdie in door embryotransplantatie geïnduceerde dooierezaktumoren in de muis, wordt mogelijk veroorzaakt door somatische celhybridisatie.

IV. De combinatie van het geslacht van het getransplanteerde embryo en de recipiënt is van invloed op de tumorsoort die daaruit ontstaat.

V. Sacrale teratomen zijn de humane tegenhanger van door embryotransplantatie geïnduceerde tumoren in de muis.

VI. Humor ten koste van zwakzinnigen keurt de samenleving af, voor het overige zijn zwakzinnigen afhankelijk van de kruimels onder de tafel.

VII. DNA onderzoek moet gebruikt kunnen worden als bewijslast bij verkrachtingszaken: de verdachten moeten verplicht kunnen worden DNA onderzoek te ondergaan.

VIII. De invloed van Mendini dient niet beperkt te blijven tot het Groninger museum: overal in de binnenstad dienen dakterrassen en plaza's aangelegd te worden.

IX. Het is aan te bevelen dat genetici naast het doen van wetenschappelijk onderzoek ook een innerlijke scholingsweg volgen.

X. Vrouwen in de politiek sluiten geen herenaccoorden, zij weten dat die leiden tot verspilling.

XI. Het verdient aanbeveling om alle auto's in Athene op zonne-energie te laten rijden.

XII. Het dragen van gebitsbeschermers en beenbeschermers tijdens een hockey-wedstrijd leidt tot spelverruwing.

XIII. De slogan "kies exact" wekt ten onrechte de suggestie dat hiermee de toekomst van meisjes verzekerd is en dient daarom vervangen te worden door: "kies precies".

XIV. Het is de hoogste tijd om te overwegen het artikel waarin wordt bepaald dat bij een huwelijk de huwelijksgoederengemeenschap van rechtswege ontstaat, uit de wet te schrappen.

XV. Het gegeven dat er nauwelijks vrouwen schaken, duidt op een onbewust verlangen bij de heren om door de dame mat gezet te worden.

Stellingen bij het proefschrift van R.J. van Berlo
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RIJKSUNIVERSITEIT GRONINGEN

THE ROLE OF SEX AND CHROMOSOMAL ABNORMALITIES IN THE DEVELOPMENT OF MURINE EMBRYO-DERIVED TUMORS

PROEFSCHRIFT

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ROMY JOHANNA VAN BERLO
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Promotores:

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Mijn dank gaat uit naar de velen die direct of indirect aan dit proefschrift hebben bijgedragen. Enkelen noem ik bij name:

Dr. B. de Jong heeft mij geïntroduceerd in de tumorcytogenetica. Zijn welwillendheid om ten alle tijden met mij in gesprek te gaan, waardeer ik zeer. Zijn humor en vertrouwen hebben mij gesteund om door te gaan.

Zonder de vele, soms temperamentvolle, leergesprekken met Prof. Dr. J. W. Oosterhuis zou dit proefschrift niet tot stand zijn gekomen. Door zijn waardevolle kritische kanttekeningen van algemene en methodologische aard, ben ik mij bewust geworden van de te betrachten zorgvuldigheid in het doen van wetenschappelijk onderzoek.

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Bij het uitvoeren van de statistische berekeningen heb ik dankbaar gebruik gemaakt van de kennis van Ir. G. J. te Meerman.

Met de niet aflatende hulp van mevrouw T. Dijkhuizen is het mogelijk geweest om meer dan 1700 karyotypen te bestuderen. Zij heeft het merendeel van de embryo's getransplanteerd en de karyotypen gelegd. Sysifus arbeid?

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

In this introduction the pathobiology of human and murine teratomas will be reviewed with respect to the usefulness of murine teratomas as models for human germ cell tumors. In the first section epidemiological and cytogenetical data on human teratomas will be discussed. The following section highlights the similarities between human and murine teratomas. In the last section studies on the factors influencing murine teratogenesis will be reviewed. Two factors investigated in this thesis are introduced: the role of chromosomal abnormalities and the possible role of the sex of the grafted embryo in the development of murine embryo-derived (ED) teratomas.

Human teratomas

Teratomas are a heterogenous group of solid tumors: incidence, clinical behavior, and morphology differ with age, sex, race, and geographical area. They occur at various anatomical sites and their composition may range from one (undifferentiated) cell type to highly organized structures such as teeth, hair, nails and fingers. This heterogeneity has led to different classification systems and, accordingly, to a number of theories to explain the histogenesis of these tumors (e.g. origin from fetal inclusions, embryonic cells, extraembryonic cells or germ cells). Today it is believed that all teratomas originate from germ cells and they are commonly referred to as germ cell tumors (GCTs). Histologically two main entities are distinguished:

1. tumors composed of neoplastic germ cells, called seminoma in the testis, dysgerminoma in the ovary, and sometimes germinoma in extragonadal sites.

2. nonseminomatous GCT, which are composed of embryonic tissues (embryonal carcinoma, immature teratoma, and mature teratoma) and/or extraembryonic tissues (yolk sac tumor, and choriocarcinoma). Nonseminomatous GCTs may have a seminoma component. Such tumors are according to the British classification called combined tumors [Pugh, 1976].

Testicular GCTs themselves are heterogeneous [see Ulbright and Roth, 1987; Mostofi *et al.*, 1987 for reviews on recent progress in pathology and immunohistochemistry]. Epidemiologically three groups of testicular GCTs can be distinguished: infantile tumors, those of adolescents and young adults, and those of elderly men. The majority of infantile testicular GCTs has the histology of yolk sac tumor [Harms and Jänig, 1986]; they are often diploid (12 out of 21 cases in a recently measured series), but may be peritetraploid [Oosterhuis *et al.*, paper in preparation]. One infantile testicular GCT (yolk sac tumor) which Oosterhuis *et al.* [1988] karyotyped lacked an iso(12p) marker chromosome. Testicular GCTs of adults can be divided into seminomas (50%) and nonseminomatous GCTs (40%), a smaller percentage combines seminoma and nonseminoma components: so-called combined tumors according to the British classification [Pugh, 1976]. Seminomas are usually hypertriploid, nonseminomatous GCTs most often hypotriploid. The components of combined tumors have the ploidy of their pure counterparts. The ploidy of these adult testicular GCTs suggests that nonseminomatous GCTs evolve through a seminoma stage by net loss of chromosomes [Oosterhuis *et al.*, 1989]. In 1982 Atkin and Baker raised the possibility that an i(12p) was a specific chromosomal change in testicular GCTs. Their observation has been confirmed by many investigators [Atkin and Baker, 1983, 1985; Gibas *et al.*, 1986; Oosterhuis *et al.*, 1986; Delozier-Blanchet *et al.*, 1987; Castedo *et al.*, 1989a, 1989b, 1989c; see for review: De Jong *et al.*, 1990]. In a study of 83 testicular GCTs De Jong *et al.* [1990] found that 69 (83%) had one or more copies of the i(12p). In about 50% of the "i(12p)-negative" tumors, Castedo *et al.* [1988] found other structural abnormalities of chromosome 12, these, possibly in concert with structural and numerical abnormalities of other chromosomes may have the same effect as the presence of i(12p). The prevalence of i(12p) was

the same in seminomas and in nonseminomatous GCTs, indicating the importance of this marker for the development of both types of adult testicular GCTs. Testicular GCTs of elderly men have the histology of spermatocytic seminoma. These tumors are negative for placental like alkaline phosphatase (PLAP) [Dekker *et al.*, paper in preparation] and not composed of gonocytes, but of more mature germ cell precursors. They are usually diploid [Müller *et al.*, 1987].

Ovarian germ cell tumors

Mature cystic teratoma of the ovary (dermoid cyst) is the most common ovarian GCT. They may rarely undergo secondary malignant degeneration, usually as squamous carcinoma. Dermoid cysts are, with very few exceptions [Parrington *et al.*, 1984] diploid with a normal female karyotype. Studies by Linder *et al.* [1975], and Parrington *et al.* [1984] have shown that they are the result of parthenogenetic activation of oocytes via at least three different mechanisms: failure of meiosis I, failure of meiosis II, or duplication of a mature ovum. Malignant tumors of the ovary occur at a younger age than dermoid cysts, usually in the second decade. The majority of the tumors has either the histology of yolk sac tumor or of immature teratoma. Data on ploidy of yolk sac tumors are scarce. Two cases of our own were aneuploid, so were 20 cases studied by Kommoss *et al.* [1990]. Speleman *et al.* [1990] karyotyped a yolk sac tumor of the ovary, they found an i(12p) marker chromosome. However this abnormality was not present in an ovarian yolk sac tumor described by Vos *et al.* [1990]. Dysgerminomas, tumors derived from dysplastic germ cell precursors, are relatively rare [Talerman, 1987]. In a recent paper on the ploidy of 25 dysgerminomas Oud *et al.* [1988] report that they are often peritetraploid (median DNA index of 1.94, range 1.25-3.23). The i(12p) chromosome has been found in two dysgerminomas of the ovary [Atkin and

Baker, 1987; Jenkyn and McCartney, 1987].

Extragonadal germ cell tumors

Extragonadal GCTs again are a heterogeneous group of tumors. They occur in the midline of the body (pineal region, hypothalamic region, anterior and posterior mediastinum, retroperitoneum and sacral area), but also away from the midline (e.g. orbit, neck, stomach, placenta etc.). The histological composition and clinical behavior of extragonadal GCTs are remarkably different depending on their anatomic localization and the sex and the age of the patient. A thorough review of fundamental, pathological and clinical aspects of these tumors is given by Gonzales Crussi [1982]. Oosterhuis *et al.* [1990] have found, that mediastinal malignant GCTs, are usually either diploid or peritetraploid, and only rarely aneuploid. Seminomas of the anterior mediastinum were either diploid or peritetraploid. Benign extragonadal GCTs are diploid, and have the same karyotype as the host [for review see Mutter, 1987]. Three nonseminomatous, malignant, mediastinal GCTs, two karyotyped by Oosterhuis *et al.* [1985, and paper in preparation] and one in the literature [Mann *et al.*, 1983], lacked i(12p). Recently i(12p) was found in two mediastinal malignant GCTs [Dal Cin *et al.*, 1989; Chaganti *et al.*, 1989]. The first pineal germ cell tumor that was karyotyped, a tumor with a nonseminomatous histology, had the i(12p) marker [Slater *et al.*, paper in preparation]. Benign extragonadal GCTs, teratomas, similarly lack i(12p) [Kaplan *et al.*, 1979]. Essentially, there are two hypotheses on the cells of origin of extragonadal GCTs: 1. the primordial germ cell theory, and 2. several nongerminal cell theories (e.g. embryonic and extraembryonic stem cells, embryonic inclusions). It is possible that extragonadal GCTs originate from different cell types, and by different pathogenesis depending on the anatomic site [Gonzales Crussi, 1982]. If this is true, it is plausible that GCTs of the mediastinum and the brain,

which have the histological spectrum of gonadal GCTs (including germinomas), arise from diploid primordial germ cells (gonocytes), which from the yolk sac have migrated along the midline of the body to sites other than the gonadal blastema. However, the other extragonadal GCTs, which consistently lack germinoma, or germinoma-components, probably originate from pluripotent embryonic or extraembryonic stem cells.

Similarities between human and murine teratomas

Human and murine teratomas have many similarities with respect to morphology, cell of origin and developmental pathways. Morphologically embryonal carcinoma (EC) cells of human and murine teratomas are almost indistinguishable. In both human and murine teratomas a variety of somatic tissues and cell types in all stages of maturation can be found. Spontaneous murine ovarian teratomas are comparable with human cystic teratomas, since they both develop from parthenogenetically activated oocytes. For human ovarian teratomas it was shown that parthenogenic activation of oocytes is a postmeiotic event [Linder *et al.*, 1975; Parrington *et al.*, 1984]. To explain his results, Linder postulated that a haploid cell underwent diploidization. Subsequently, Eppig *et al.* [1977] demonstrated the same for spontaneous ovarian teratomas in LT-strain mice. Both in mice and in humans these tumors are almost always benign. Another similarity between murine ovarian teratomas and human cystic teratomas is that both initiate after birth. In mice ovarian teratomas develop from about 30 days of age, and in humans cystic ovarian tumors arise after puberty. Thus both could be under hormonal control. Murine testicular teratomas and their human counterparts originate from premeiotic germ cells. Spontaneous murine testicular teratomas are benign, whereas in adolocents and young men these tumors are almost always malignant. Murine testicular teratomas are fetal in origin. In prepubertal boys with abnormal

gonadal development carcinoma in situ has been found [Müller and Skakkebaek, 1984; Cortes *et al.*, 1989], this indicates that human germ cell tumors start their development during intrauterine life.

Murine teratomas as a model for human teratomas

Teratomas in animals could provide relevant information to the understanding of the biology of human teratomas. However, spontaneous teratomas are rare in most animal species with the exception of birds, horses and certain mouse strains. Since almost all experimental work has been performed with teratomas originating in mice, the following will be confined to these. The stem cells of benign and malignant murine teratomas, the embryonal carcinoma (EC) cells are pluripotent: they are capable of forming derivatives of all three germ layers, and can proliferate in the undifferentiated state. Morphologically they show a striking resemblance with the cells of an early embryo. Tumors in which all EC cells are differentiated (or have died) are called teratomas and they are considered benign. Malignant teratomas, known as teratocarcinomas, are composed of a teratoma component and EC cells. Contrary to teratomas, teratocarcinomas are retransplantable. Retransplantation may result in the development of a benign tumor, or in teratocarcinomas predominantly composed of one cell type (e.g. only EC cells, or one differentiated cell type that probably has undergone malignant transformation, the most common example is yolk sac carcinoma) [Martin, 1975].

Testicular teratomas were first observed in 1% of strain 129 mice [Stevens and Little, 1954]. Selection of inbred sublines ultimately resulted in an incidence of testicular teratomas of 30% [Stevens, 1973]. These tumors can be recognized on day 15 of gestation as clusters of undifferentiated embryonic cells within the seminiferous tubules [Stevens, 1962]. Ultrastructural similarities between EC cells and primordial germ cells [Pierce and Beals, 1964; Pierce *et*

al., 1967] suggests that testicular teratocarcinomas arise from primordial germ cells. Ovarian teratomas occur with an incidence of about 50% in females of the mouse strain LT, [Stevens and Varnum, 1974]. They originate, starting at about 30 days of age, from ovarian oocytes that develop parthenogenetically. At first, the parthenogenetic embryos resemble normal ones, but at the blastocyst stage most become disorganized. Parthenogenesis has also been observed in a small percentage of ovulated LT eggs. After cleavage they implant in the uterus after which most die at five to seven days of gestation. Analogous to some murine tumors of viral origin [Rowe, 1973], mutant genes could be directly responsible for tumorigenesis in 129 and LT mice. However, as pointed out by Mintz and Fleischman [1981], mutant genes might cause spontaneous initiation of parthenogenetic cleavage in ovarian oocytes in LT females and fetal germ cells in 129 males.

The study of murine teratomas was greatly facilitated by the discovery that the incidence of tumor formation could be increased by transplanting either genital ridges or early embryos to ectopic sites. Testicular teratomas can be induced by grafting male genital ridges from 12.5-day mouse fetuses into the testes of syngeneic recipients (strain 129) [Stevens, 1964]. The incidence of teratomas, induced by this method, is about 80%. Transplantation of female genital ridges of the same age do not yield teratomas. This is not surprising since their germ cells have entered the prolonged meiotic profase, and thus incapable of undergoing parthenogenesis. Teratomas can also be induced by transplanting pre- or postimplantation mouse embryos to the testes [Stevens, 1970a] or under the kidney capsule [Stevens, 1970b; Damjanov *et al.*, 1971] of syngeneic recipients. These embryo-derived (ED) tumors, which are indistinguishable from spontaneous teratomas, develop either benign or malignant. It could be argued that ED tumors originate from germ cells present in the grafted embryo rather than from pluripotent embryonal cells. To investigate this possibility Mintz *et al.* [1978] transplanted 6 day old embryos of genetically sterile genotypes (i.e. W/W or SL^J/SL^J) into the testes of syngeneic

recipients. They obtained, as readily as in genetically normal controls, teratocarcinomas composed of undifferentiated EC cells and differentiated derivatives.

Since murine ED tumors are indistinguishable from spontaneous murine teratomas, most experimental work has been done with these teratomas and with cell lines derived from spontaneous and induced tumors. The induction of teratomas by embryo transplantation does not only result in a very high yield of teratomas, benign and malignant, but also in the yield of malignant female tumors. Besides this method can be applied to mouse strains that do not develop teratomas spontaneously. The ratio of benign to malignant ED tumors varies from one inbred strain to another [Solter *et al.*, 1979; Damjanov *et al.*, 1983]. Strains with a yield of 50% or more teratocarcinomas are called permissive strains, whereas in nonpermissive strains only 10 to 15% of the transplanted embryos develop into a malignant tumor. A number of embryo- and host-related factors has been investigated to establish their role in ED teratocarcinogenesis [Solter *et al.*, 1979, 1980, 1981; Damjanov *et al.*, 1982; Damjanov and Solter, 1982]. Although the results of these studies showed that genetic and epigenetic factors influence the malignant transformation of an embryo transplanted to an extrauterine site, they also showed that permissiveness nor nonpermissiveness are absolute entities. Because manipulation of the embryo or the host can modify the yield of teratocarcinomas it seems as if ED teratocarcinogenesis is regulated by positive and negative stimuli. It is only by chance that one of these stimuli will prevail. According to Damjanov *et al.* [1983] this is why in most mouse strains a 50% yield of teratocarcinomas is obtained from transplanted embryos.

Thus far no systematic cytogenetic investigations of (primary) ED tumors have been performed. Karyotyping might provide a better understanding of two factors in the malignant development of ED tumors: the role of chromosomal abnormalities and the possible role of the sex of the grafted embryo. As has been mentioned above, a wealth of cytogenetic data of human germ cell tumors

has been accumulated. Aberrations of chromosome 12 as well as polyploidy seem to play an important role in the pathogenesis of these tumors. It is possible that chromosomal abnormalities comparable to those found in human teratomas are present in murine teratomas as well. Moreover, cytogenetic studies of ED teratomas, could reveal differences between the malignant development of male and female teratomas, and thus contribute to a better understanding of the malignant development of human teratomas. Attempts to determine the sex of embryos before transplantation have not been described. However, karyotype analysis of ED tumors could provide indirect information of the sex chromosomal constitution of transplanted embryos. It is a salient fact of the epidemiology of human teratomas that although teratomas are more common in females, the proportion of malignant tumors is higher in males both in the gonads and in extragonadal sites [O'Hare, 1978; Gonzales Crussi, 1982; Gilman, 1983]. For example, data from John Hopkins Hospital obtained from all discharge diagnoses between January 1953 through June 1977 showed that 25% of teratomas occurred in males of which 86% were malignant. On the other hand, only 11% of 174 teratomas in females were malignant. The resulting absolute number of malignant tumors in males being more than twice that in females [Gilman, 1983]. The importance of sex chromosomal constitution in the pathogenesis of malignant germ cell tumors of the gonads is further illustrated in patients with gonadal dysgenesis. Patients with a 46,XX or 45,X karyotype very rarely have gonadoblastomas or germ cell tumors. On the other hand these tumors often complicate gonadal dysgenesis associated with 46,XY karyotype, a 45,X/46,XY karyotype, and other mosaic karyotypes that include a Y chromosome [Scully, 1979]. Among woman with 46,XY gonadal dysgenesis, germ cell tumors develop almost exclusively in individuals with H-Y^+ phenotype [Warner *et al.*, 1985]. An interesting observation in this context is the high incidence of partial hydatiform moles in abortuses with male genetic constitution [Jacobs *et al.*, 1982]. Murine teratocarcinomas constitute the most readily available model for human malignant teratomas [Damjanov *et al.*, 1983].

Results from a study on the role of the sex of the transplanted embryos in the regulation of evolving malignancy in ED tumors might contribute to a better understanding of the aforementioned problems in the biology of human teratomas.

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CHAPTER II

YOLK SAC CARCINOMA DEVELOPS SPONTANEOUSLY AS A LATE OCCURRENCE IN SLOW GROWING TERATOID TUMORS PRODUCED FROM TRANSPLANTED 7-DAY MOUSE EMBRYOS

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ABSTRACT

Seven day embryos of BALB/c mice transplanted underneath the kidney capsule of adult syngeneic recipients form either benign teratomas or teratocarcinomas, which can be distinguished from one another histologically at 8 weeks post-embryonic transplantation. Embryo derived (ED) teratomas were allowed to remain in the host for an additional period up to one year after embryo transplantation to test their malignant potential. It was found that a considerable number of slow growing small tumors derived from embryonic transplant give rise to parietal yolk sac carcinomas. A proportion of these tumors contained foci of visceral yolk sac and trophoblastic differentiation, which gradually disappeared in successive transplantations. We conclude that parietal yolk sac carcinoma develops as a late event in some ED teratomas. These malignant tumors originate either from small foci of yolk sac originally included in the grafted embryo or, more likely, from the yolk sac formed from the differentiating embryonic stem cells.

INTRODUCTION

ED teratomas and teratocarcinomas have been extensively studied by Solter *et al.* [1979, 1980, 1981] and Damjanov *et al.* [1982, 1983] who showed that the outcome of embryonic transplantation and the nature of the tumor depend on embryonic as well host related factors. In all those experiments the results of embryonic transplantation were reviewed at 8 weeks, with the assumption that at that time interval one can confidently distinguish the benign teratomas, which are usually small and weigh 1 to 2 g, from the larger malignant tumors which weigh over 5 g. However, the malignant potential of small tumors which, at 8 weeks post-transplantation of the embryos, weighed less than 2 to 5 g, was never systematically evaluated.

In the present study we have evaluated the ED teratiod tumors before 8 weeks and long-term by prolonging the observation period beyond the originally recommended initial 8 weeks.

We report that under these experimental conditions it would be expected that fewer malignant tumors would be harvested than if the embryonic grafts were removed at 8 weeks, suggesting that some of the "malignant" tumors are actually benign but slower maturing teratomas. Furthermore, we show that the benign teratomas left in the recipients of embryonic grafts continue to grow and could give rise to transplantable yolk sac carcinomas.

MATERIALS AND METHODS

Seven day old BALB/c mouse embryos were isolated from the uteri of dated pregnant females. The day the vaginal plug was found was labelled day 0. The embryos were isolated, dissected free from extra-embryonic tissue and the Reichert's membrane, and transplanted under the kidney capsule of syngeneic male and female recipients using the technique of Damjanov *et al.* [1988].

The mice were killed when the tumor reached a size of 1.5 to 2.5 cm, or arbitrarily at 220 to 365 days after embryo transplantation. Representative samples of primary ED tumors were taken for transplantation and histology.

To assess transplantability, primary ED tumors larger than 6 mm in diameter were transplanted intramuscularly in the left hind leg of syngeneic recipients. Transplantable tumors were further retransplanted at least four times.

All tumors were histologically investigated. Paraffin embedded sections were made and stained with hematoxylin and eosin. Malignant tumors (teratocarcinomas) are histologically characterized by presence of embryonal carcinoma (EC) cells [Stevens and Pierce, 1975], whereas all tumors without EC cells are considered benign (teratomas).

Trophoblastic components were identified by enzyme histochemical staining for alkaline phosphatase according to Gomori [1952], and for 3-ß-hydroxysteroid dehydrogenase according to Allen [1960].

RESULTS

A total of 101 embryos were transplantated to either male or female BALB/c mice. The take rate was 76% (n=41) in male recipients and 86% (n=40) in female recipients. In male recipients, 19 of ED tumors were classified as teratocarcinomas, in the females 17. The teratocarcinomas were all harvested within 200 days after embryo transplantation (Table 1), on the basis of the size of the tumor.

Table 1: Histology of embryo-derived tumors

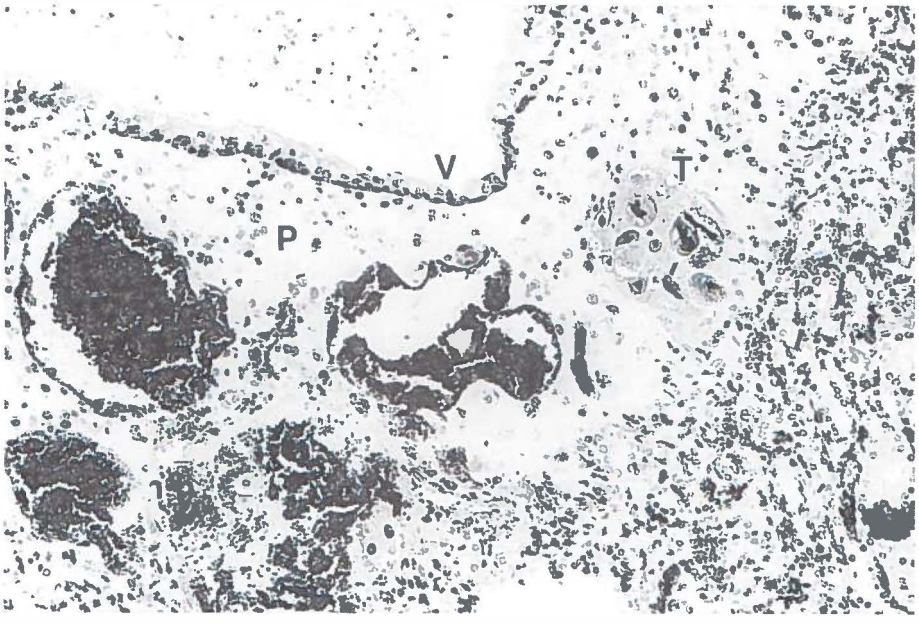
histology	in male recipients			in female recipients		
	number	range[1]	mean[1]	number	range[1]	mean[1]
teratocarcinomas	19	50-117	67	17	73-198[2]	106[2]
teratomas	8	266-363	298	10	224-254	237[3]
PYS ± VYS ± T ± Tr[4]	14	176-348	250	13	225-254	237

[1] days after which the mice were killed
[2] data of 15 tumors, two mice died spontaneously
[3] data of 9 tumors, one mouse died spontaneously
[4] PYS, parietal yolk sac component; VYS, visceral yolk sac component; T, teratoma component; Tr, trophoblastic component

Histological examination of tumors arbitrarily harvested 200 to 365 days after

transplantation disclosed two types (Table 1): a. teratomas composed of somatic tissue only, which were consistently small (n=18, 8 in males and 10 in females), and b. those composed of parietal yolk sac (PYS) tumor (n=27, 14 in males and 13 in females), most often combined with visceral yolk sac (VYS) elements (21/27), and teratoma (24/27), and sometimes with trophoblastic elements (7/27). The latter tumors were of varying size. In the tumors smaller than 1 cm in diameter, the yolk sac component typically formed a small proportion of the tumor tissue and one tumor contained scattered throphoblastic giant cells in (Fig. 1). The trophoblastic nature of the giant cells

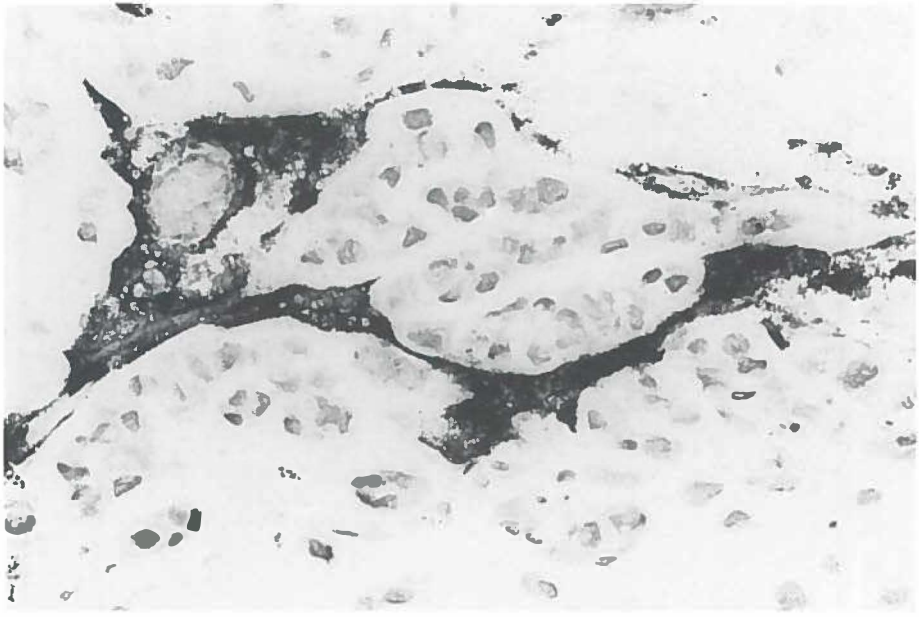
Figure 1



Primary ED tumor harvested after >200 days of follow up: small tumor composed of PYS, VYS, and trophoblastic giant cells. In the right lower corner the renal parenchyma is visible (hematoxylin and eosin, x 140).

was confirmed by positive staining for alkaline phosphatase, and 3-ß-hydroxysteroid dehydrogenase (Fig. 2). The larger tumors (9 tumors, 1 to 1.5 cm in diameter) were predominantly composed of PYS tumor, usually accompanied by VYS (7/9) (Fig. 3), teratoma (6/9), and groups of throphoblastic cells in a hemorrhagic background (6/9) (Fig. 4). The size of the tumor and the predominance of the yolk sac component suggest that these tumors started proliferating after an initial latent dormant period.

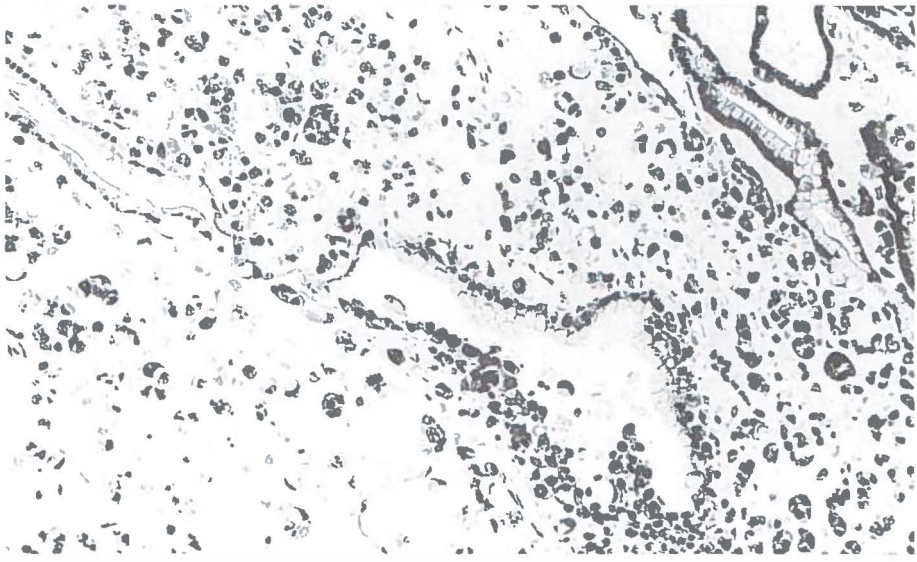
Figure 2



Trophoblastic giant cells in PYSC staining positively in the Gomori stain for alkaline phosphatase (x 350).

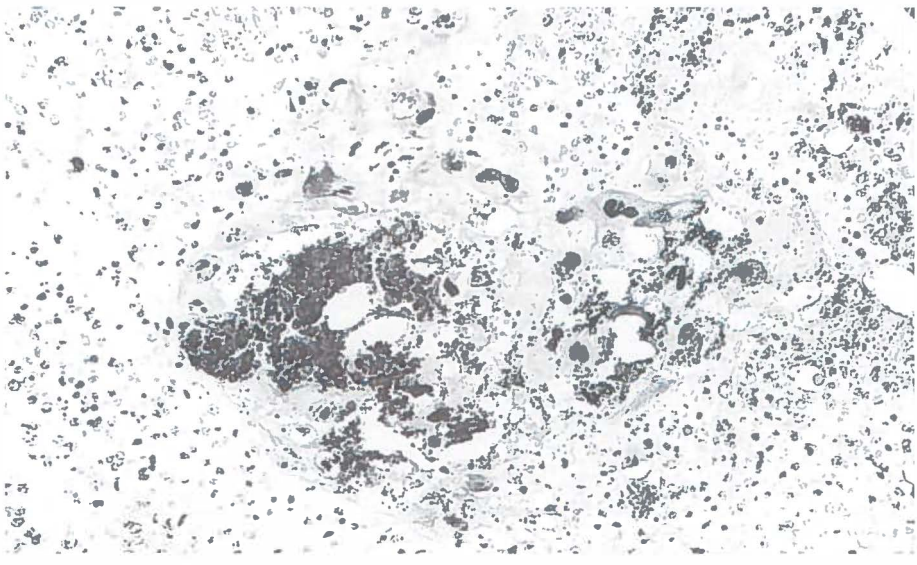
To assess transplantability all primary ED tumors larger than 6 mm. in diameter were transplanted intramuscularly in the left hind leg of adult BALB/c

Figure 3



Primary ED tumor harvested after > 200 days of follow up: large tumor composed of PYS and VYS (hematoxylin and eosin, x 140).

Figure 4



Group of trophoblastic giant cells in a PYSC embedded in a hemorrhagic background (hematoxylin and eosin, x 140).

mice. Table 2 shows the results of transplantation. Thirty-four of the 36 primary teratocarcinomas were transplanted and 29 proved to be retransplantable for at least 4 generations. Only 15 out of the remaining 45 tumors were, at harvest, large enough to be transplantable. Seven of these tumors could be transplanted for at least 4 generations, all of them were larger than 1.5 cm in diameter, and predominantly composed of PYS tumor. The VYS-, teratoma-, and trophoblastic components present in the primary ED tumors disappeared in the course of three retransplantations and were absent in the fourth passage. In view of their morphology and transplantability these tumors should be labeled as PYS carcinomas (PYSC).

Table 2: Results of retransplantation of primary ED tumors

histology of the primary tumor	number	retransplanted	transplanted for at least 4 generations
teratocarcinoma			
male recipients	19	19	17
female recipients	17	14[1]	12
teratoma			
male recipients	8	0	-
female recipients	10	1	0
PYS ± VYS ± T ± Tr[2]			
male recipients	14	6	4[3]
female recipients	13	8	3[3]

[1] one tumor was not available, two mice died spontanously
[2] PYS, parietal yolk sac component; VYS, visceral yolk sac component; T, teratoma component; Tr, trophoblastic component
[3] All transplantable tumors were derived from slow growing, large primary ED tumors, predominantly composed of PYS. After three transplantations the tumors were pure PYS carcinomas

DISCUSSION

We report the novel observation that after a long period of latency PYSCs reproducibly develop from seven day old BALB/c mouse embryos grafted under the kidney capsule of syngeneic mice. Beyond day 200 after embryo transplantation teratocarcinomas were never observed, but growing tumors were always predominantly composed of PYSC. The tumors are similar to the three PYSCs described by Damjanov and Solter [1973], which were also harvested after long observation, at least 5 months after the embryo transplantation (not shown).

Yolk sac carcinoma in mice may be obtained from teratocarcinomas by cloning or by spontaneous differentiation [Pierce *et al.*, 1962; Sherman and Miller, 1978]. Murine EC cells can be induced to yolk sac differentiation by exposure to retinoic acid [Strickland and Mahadavi, 1978]. These yolk sac tumors are derived from EC cells, and contain only PYS. Sobis *et al.* [1983] produced yolk sac carcinomas composed of both PYS and VYS from displaced yolk sacs after fetectomy in 129 Sv/Sl mice. The carcinomas do metastasize, the metastases containing both components of the primary tumor. The tumors probaby develop from stem cells that are either present in the endoderm of the visceral yolk sac at the time of operation, or appear afterwards by dedifferentiation [Sobis *et al.*, 1983]. These yolk sac carcinomas of extra-embryonic derivation also took a long time, eight to nine months, to develop.

The primary ED tumors from which the transplantable PYSCs were derived contained VYS and trophoblastic elements in addition to teratoma, but these components were gradually lost in three transplantations, and had disappeared by the fourth passage. Apparently these components are outgrown by the malignant PYSC cells. Trophoblastic elements are rare in murine teratocarcinomas. Stevens [1960] also noted that trophoblastic giant cells disappeared after successive transplantations of a testicular mouse teratocarcinoma. The only transplantable mouse tumor cell line with

trophoblastic differentiation is the E6496 cell line, established from a spontaneous ovarian teratocarcinoma [Fekete and Ferigno, 1952; Damjanov *et al.*, 1985]. Rat yolk sac carcinoma derived from displaced yolk sac [Sobis *et al.*, 1982], as opposed to ED rat yolk sac carcinoma [Damjanov and Sell, 1977; Damjanov *et al.*, 1977], does have a transplantable trophoblastic component. Sobis *et al.* [1982] dismiss the possibility that the trophoblastic cells are derived from cells originally attached to the Reichert's membrane, since these cells are terminally differentiated, and thus unable to grow. They conclude that after displacement of the visceral yolk sac, a population of multipotential cells appears that may give rise to all embryonal and extra-embryonal structures. In our tumors the trophoblastic giant cells were most often found in the larger tumors and entirely surrounded by PYS. Therefore, it is unlikely that we are dealing with contaminating cells from the original graft.

The cell of origin of the late appearing ED PYSCs cannot be determined from this study. Theoretically it is possible that small fragments of parietal endoderm adhering to the surface of the egg cylinder, and grafted together with the embryos, gave rise to the tumors. Alternatively, the PYSCs are derived from EC cells that underwent extra-embryonic differentiation, as it is well known that ED teratocarcinomas may contain yolk sac. Conceivably in the ED tumor model, EC cells and their embryonic derivatives are the most rapidly growing cells. When they undergo terminal differentiation to become mature teratoma, extra-embryonic cells may become the dominant cell type, probably only after malignant transformation. Cytogenetic data support the malignant character of the PYS cells (not shown). The PYSC outgrows the VYS and trophoblastic components. Cloning of these cells might results in tumors with a stable VYS- or trophoblastic phenotype.

We speculate that our model of late appearing ED yolk sac tumor preceded by teratoma probably has its human counterpart in sacral teratoma, which after the initial diagnosis of benign teratoma, may recur as yolk sac tumor [Gonzales

Crussi, 1982], sometimes after a long latency period.

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CHAPTER III

CYTOGENETIC ANALYSIS OF MURINE EMBRYO-DERIVED TUMORS

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ABSTRACT

The possible relationship among malignancy, differentiation and chromosomal constitution of primary embryo-derived tumors was studied. Tumors were induced by transplanting seven-day-old mouse embryos under the kidney capsule of syngeneic BALB/c recipients. Transplantation of 101 embryos resulted in 81 tumor-bearing mice: 36 teratocarcinomas, 18 teratomas and 27 yolk sac tumors. Some of the yolk sac tumors proved to be retransplantable for several generations.

Cytogenetic investigation of the primary embryo-derived tumors revealed that the majority of teratocarcinomas (82%) was chromosomally normal, whereas almost all (83%) karyotyped teratomas and yolk sac tumors had a highly abnormal chromosomal constitution. Most common aberrations were: polyploidy; overrepresentation of chromosome 1, 6, 15 or 19; and an underrepresentation of chromosome 2, 4, 14, or a sex chromosome.

INTRODUCTION

In most inbred mouse strains teratomas can be obtained experimentally by grafting pre- or postimplantation embryos to extra-uterine sites [Stevens, 1970a, 1970b; Damjanov *et al.*, 1971]. The resulting tumors are indistinguishable from spontaneously occurring ovarian and testicular teratomas, and they are referred to as embryo-derived (ED) teratomas. The stem cells of murine teratomas, the embryonal carcinoma (EC) cells are pluripotent; they are capable of forming derivatives of all 3 germ layers and can continue to proliferate in the undifferentiated state. Tumors in which all EC cells are differentiated (or have died) are called teratomas and they are considered benign. Teratocarcinomas are composed of a teratoma component and EC cells, and are considered malignant. In addition ED tumors may develop along extra-embryonic lineage

to form yolk sac tumors [Martin *et al.*, 1978].

We recently described the histological and biological findings of 101 embryo transplantations [Van Berlo *et al.*, 1990]. In total, 36 ED tumors contained EC cells and were classified as teratocarcinomas. The 45 ED tumors without EC cells, could be histologically subdivided into 2 groups: those composed of somatic tissue only and those composed of parietal yolk sac (PYS) tumor, most often combined with visceral yolk sac (VYS) elements, teratoma (T), and sometimes with trophoblastic (Tr) elements. Surprisingly some tumors of the latter group proved to be retransplantable for several generations.

The purpose of this study was to investigate whether chromosomal abnormalities are present in primary ED tumors and to determine their possible role in oncogenesis.

MATERIALS AND METHODS

Tumors were induced by transplanting seven-day-old embryos under the kidney capsule of adult male and female BALB/c recipients using the technique described by Damjanov *et al.* [1987]. The mice were killed when the tumor reached a size of about 1.5 to 2.5 cm or randomly during the 225- to 365-day period after embryo transplantation. Representative samples of primary ED tumors were taken for retransplantation, histology and tissue culture in order to analyze the chromosomal constitution of the tumors.

Paraffin embedded sections of all tumors were made and stained with hematoxylin and eosin. On the basis of the criteria and nomenclature proposed by Stevens and Pierce [1975] all tumors were histologically classified as either teratocarcinoma, teratoma or yolk sac tumor which always contained PYS ± VYS ± T ± Tr [Van Berlo *et al.*, 1990].

For culture fresh tumor tissue was disaggregated by mincing with scissors

and incubating in 0,8% collagenase II (Worthington Diagnostic Systems Inc., Freehold NY). The disaggregated tumor cells were washed once with culture medium: RPMI-1640 (Gibco) supplemented with 15% fetal calf serum (Seralab), 2mM glutamine (Gibco), 100 unit/ml penicillin (Gibco), and 100 ug/ml streptomycin (Gibco), seeded in T75 tissue culture plastic flasks (Corning) and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. The next day the medium was removed, and centrifuged and 50% of it returned together with 50% fresh medium. Until harvesting, the cultures were maintained by partly changing the medium and, when necessary, by passaging.

Chromosomal preparations were made by conventional methods, and G banding was performed by a slight modification of the method used by Wang and Fedoroff [1972]. Chromosomes were classified using descriptions made by Cowell [1984] and arranged according to the standard mouse karyotype [Committee on Standardized Genetic Nomenclature for Mice, 1972]. To describe structural abnormalities the Nesbitt and Francke nomenclature [1973] was used.

According to standard rules, an abnormality is considered clonal if it is found in at least 2 or 3 tumor karyotypes: the first criterion is used for extra copies of a chromosome and structural abnormalities; the second criterion is used for missing copies of a chromosome. To determine numerical abnormalities in polyploid cells, the number of copies present was compared with the expected number of copies (e.g. in a 2N cell, one would expect 2 copies; in a 3N cell, 3 copies, and so on). The ploidy level of a cell was determined using the following, arbitrary, classes: 30 to 49 chromosomes = 2N; 50 to 69 chromosomes = 3N; 70 to 89 chromosomes = 4N; 90 to 109 chromosomes = 5N; and 110 to 129 chromosomes = 6N.

Flow cytometry was performed on frozen tissue of primary and/or retransplanted ED tumors. Nuclei were isolated using a detergent-trypsin method and stained with propidium iodide [Vindeløv *et al.*, 1983]. Measurements were obtained with the FACS 440 flow cytometer (Becton

Dickinson). As an internal standard trout red blood cells were added to the suspensions.

RESULTS

As described 101 embryos were transplanted under the kidney capsule of syngeneic male and female BALB/c recipients [Van Berlo *et al.*, 1990]. All ED tumors were histologically classified as either teratocarcinoma, teratoma or yolk sac tumor. In total 36 teratocarcinomas, 18 teratomas and 27 yolk sac tumors were found. Some of the yolk sac tumors proved to be retransplantable for several generations.

Karyotyping was performed after culture of the primary ED tumors, and usually 10 metaphases were examined. The results of cytogenetic analysis are summarized in Table 1. All teratocarcinomas harvested from a male recipient could be karyotyped, whereas 2 teratocarcinomas which arose in a female recipient were not available for cytogenetic analysis. All, except one, teratocarcinomas that developed in a male recipient had a normal chromosomal constitution. Two karyotypes of the aberrant teratocarcinoma showed polyploidy. Cytogenetic analysis of teratocarcinomas, induced in female recipients, was more difficult because of poor banding quality and/or a low yield of metaphases. In 5 of 15 teratocarcinomas consistent chromosomal abnormalities were found (Table 2). Loss of a Y chromosome was found in 2 different tumors (E84-207 and E84-455). An extra copy of an autosome was found in 2 other tumors: a trisomy 19 in tumor E84-166 and 3 copies of chromosome 8 (an isochromosome and a normal copy) in tumor E84-206. The structurally abnormal chromosome 5 found in another teratocarcinoma (E84-476) is probably the result of a translocation or a duplication.

Teratomas were always small and therefore difficult to culture. Yolk sac tumors were of varying sizes, and only the larger tumors could be cultured. In

Table 1: Cytogenetic analysis of primary ED tumors

abnormalities	histological number n	number of tumors karyotyped n	%	number of tumors with clonal n	%
in male recipients					
teratocarcinomas	19	19	100%	1	5%
teratomas	8	2	25%	2	100%
PYS ± VYS ± T ± Tr[1]	14	8	57%	7	88%
in female recipients					
teratocarcinomas	17	15	88%	5	33%
teratomas	10	1	10%	1	100%
PYS ± VYS ± T ± Tr[1]	13	1	7%	0	0%

[1] PYS, parietal yolk sac component; VYS, visceral yolk sac component; T, teratoma component; Tr, trophoblastic component

Table 2: Karyotype analysis of primary ED teratocarcinomas with an abnormal chromosomal constitution (6/34)[1]

tumornumber	recipient	abnormality	found in[2]
E84-166	female	+19	5/13
E84-206	female	iso(8)	2/4
E84-207	female	-Y	9/11
E84-455	female	-Y	4/8
E84-476	female	5q+	5/10
E86-426	male	polyploidy	2/6

[1] all 34 tumors were cultured less than 45 days
[2] number of cells with that abnormality as a fraction of the karyotyped cells

total 3 of 18 teratomas and 9 of 27 yolk sac tumors could be karyotyped. Contrary to the teratocarcinomas, almost all (10 of 12) karyotyped primary ED tumors without EC cells, were cytogenetically abnormal. The chromosomal abnormalities of the teratomas and yolk sac tumors are summarized in Table 3 and 4, respectively. All aberrant teratomas (n=3) and yolk sac tumors (n=7) were extremely heterogeneous, and polyploidy was a common phenomenon. The ploidy level differed considerably among and within the different tumors (ploidy levels of about 3N, 4N, 5N, and even 6N were observed), making the analysis of numerical abnormalities very difficult. Considering all ploidy levels within a tumor, an overrepresentation of one or more of the following chromosomes (Nos. 1, 6, 15, and 19) was found in 7 tumors, whereas one or more of the following chromosomes (Nos. 2, 4, 14, and a sex chromosome) were underrepresented in 7 tumors. Although other chromosomes were also found to be over- or underrepresented in several tumors, the mentioned ones are, in our view, of special interest because chromosomes 1 and 15 were never found to be clonally underrepresented in a tumor; chromosomes 2, 4, and 14 were never found to be clonally overrepresented in a tumor; and the numerical abnormalities of chromosomes 6, 19 and a sex chromosome were found through all ploidy levels (including 2N cells) and described as aberration in teratocarcinomas.

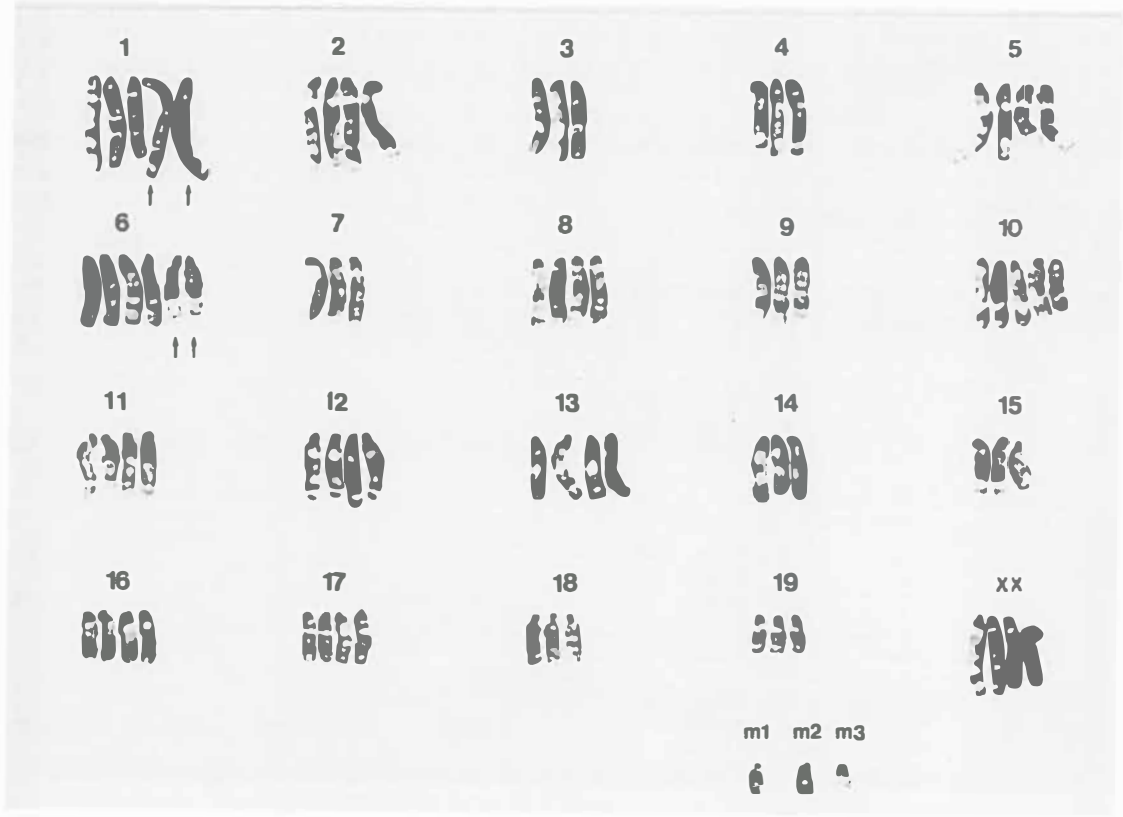
Structural abnormalities involving one or more of the following chromosomes (Nos. 1, 4 and 13) were found in several tumors. Clonal structural abnormalities of chromosome 1 were found in 2 tumors (one teratoma, and one yolk sac tumor; Figs. 1 and 2, respectively); in another 4 nonclonal abnormalities were found. In all 6 tumors the abnormal chromosome 1 was found in addition to the expected number of copies of chromosome 1. One teratoma and 2 yolk sac tumors had a clonal structural abnormal chromosome 4, with breakpoints in Bands D, E (see Fig. 3), and E2 (see Fig. 2), respectively. In another 2 cases nonclonal abnormalities were found with breakpoints in Bands D and E2. The presence of an abnormal chromosome 4

Table 3: Karyotype analysis of primary ED teratomas with an abnormal chromosomal constitution (3/3)

tumornumber	recipient	harvest[1] in vivo	harvest[1] in vitro mean	harvest[1] in vitro range	ploidy[2] N	ploidy[2] range	ploidy[2] n	clonal abnormality[3] structural	clonal abnormality[3] numerical
E87-33VIII	male	288	150	129-163	4N	75-81	8	t(1;?)(H;?) (7) m (2) f (8)	-3(4), -4(7), -5(3), +6(3), -7(5), -9(7),+ 10(3), +12(2), -13(4), -14(4), -19(4)
E87-34	male	363	66	9-91	2N 3N 4N	39-44 69 77	7 1 1		+1(3), +10(2), +15(2),+ 17(2), +18(3), +19(3)
E87-36	female	252	60	23-70	2N 4N	38-40 74	11 1	del(4)(D) (3)	-4(3)

[1] harvest time in days
[2] N, ploidy level of cells determined by using arbitrary classes; range, chromosomenumbers found in that tumor for that ploidy level; n, number of cells of that ploidy level
[3] over- and underrepresentation considering all ploidy levels of that tumor; between brackets are the number of cells in which the abnormality was found

Figure 1



G-banded karyotype of teratoma E87-033VIII, arrows indicate aberrant chromosomes.

Table 4: Karyotype analysis of primary ED yolk sac tumors with an abnormal chromosomal constitution (7/9)

tumornumber	histology[1]	recipient	harvest[2] in vivo	harvest[2] in vitro mean	harvest[2] in vitro range	ploidy[3] N	ploidy[3] range	ploidy[3] n	clonal abnormality[4] structural	clonal abnormality[4] numerical
E86-580	TPVTr	male	186	65	65	2N 4N 5N	42-46 87-88 90-92	4 2 3		+1(7), -2(4), -3(3), -4(4), -5(3), +6(9)*, -8(3), -9(4), -10(4),+11(8)*, +12(9)*, -13(3), -14(4)*, -16(4), -17(3), -18(3), +19(7), -S(9)*
E86-874	PVTr	male	220	129	46-157	2N 3N 5N	39-41 53-57 94	3 7 1		+1(5), -2(8), -3(7), -4(8), +5(4), -7(6), -13(6), -14(6),-16(8), -18(6), +19(3), +S(7)*
E86-875	PVTr	male	220	183	161-188	2N 3N 4N	45 52-68 7	1 11 11	ι(13;?)(D;?) (2)	+1(11)*, -2(6), +3(4)*, -3(5), -4(7), +5(9), +6(8)*, +7(3), +8(4), -8(4), 9(3), -9(6), +10(8)*, +11(6), -12(9), +13(2), -13(5), -14(5), +15(8), +16(3), -16(3), +17(6), +18(2), -18(3), +19(2), -19(3), +S(3), -S(5)
E86-893	TPTr	male	239	92	63-99	4N	74-82	10	rob(1;12) (2) der(4) ι(1;4) (D;E2) (3) ι(13;?)(D2;?) (2) rob(18;?19) (2) m (2) f (2)	-3(5), -4(7), -5(3), +6(2), -7(6), -8(6), +9(2), -9(3), -12(4), -14(3), +15(2), +19(2), -S(6)

Table 4: Karyotype analysis of primary ED yolk sac tumors with an abnormal chromosomal constitution (7/9) -continued-

tumornumber	histology[1]	recipient	harvest[2] in vivo	harvest[2] in vitro mean	harvest[2] in vitro range	ploidy[3] N	ploidy[3] range	ploidy[3] n	clonal abnormality[4] structural	clonal abnormality[4] numerical
E86-905	TP	male	244	115	91-127	2N	40	3	t(4;?)(E;?) (6)	-2(3), +3(2), +6(4), -7(4), -8(3), +10(2),
						4N	73-75	2	del(12)(D) (4)	-11(4), -12(7), -14(3)*, +15(4)*, -16(3),
						6N	114-117	2	m (2)	-17(3), -S(3)
E87-35	TPV	male	244	38	29-49	2N	39-40	9		
						4N	39-40	2		
E87-42IV	TPV	female	252	53	43-71	2N	39-40	6	f (2)	
						4N	76-80	5		

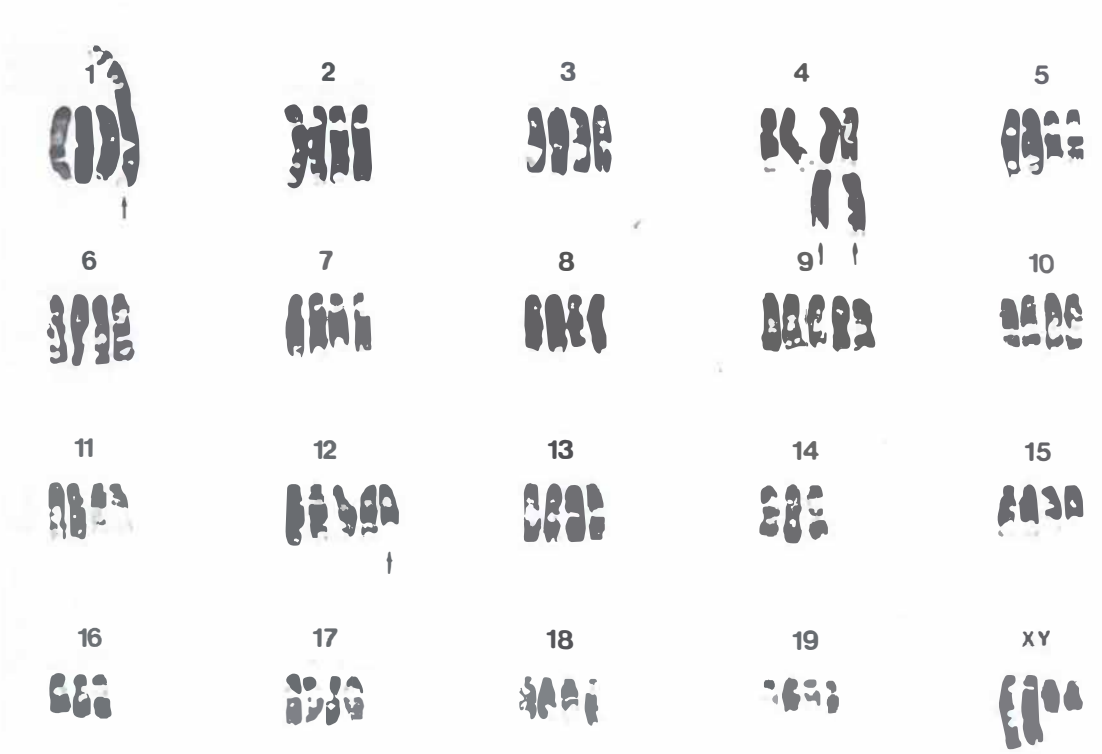
[1] T, teratoma component; P, parietal yolk sac component; V, visceral yolk sac component; Tr, trophoblastic component

[2] harvest time in days

[3] N, ploidy level of cells determined by using arbitrary classes; range, chromosomenumbers found in that tumor for that ploidy level; n, number of cells of that ploidy level

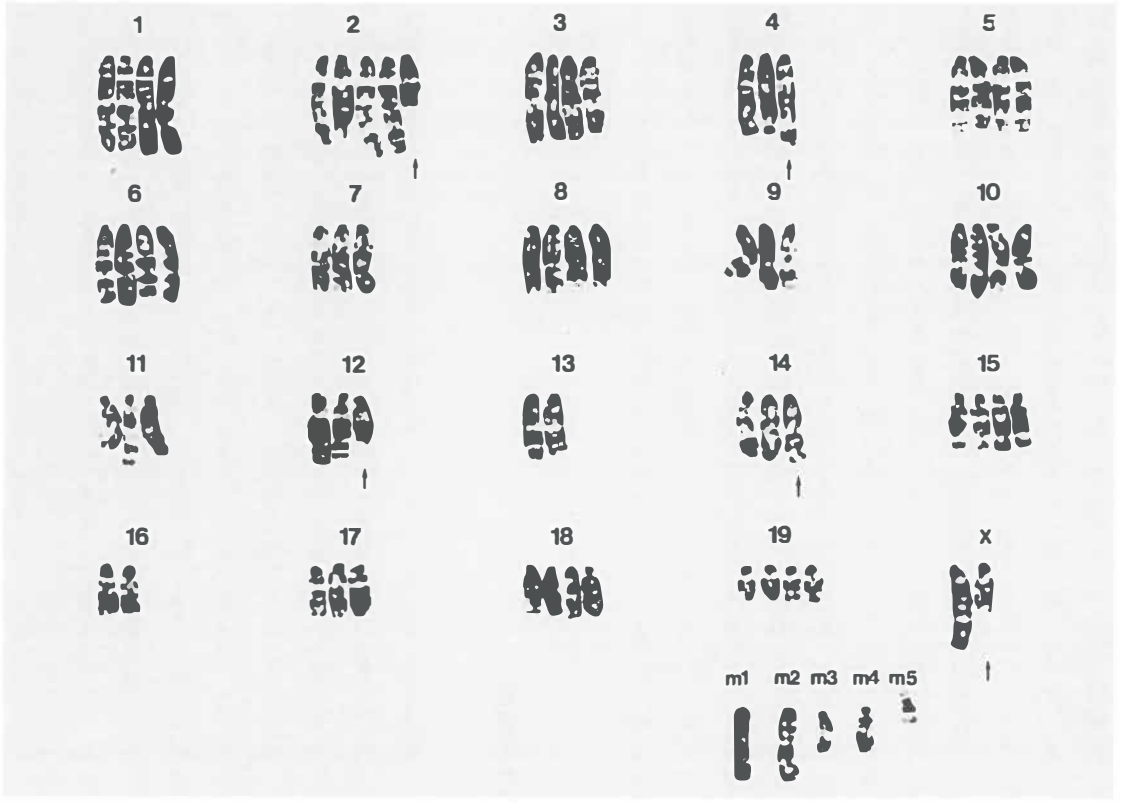
[4] over- and underrepresentation considering all ploidy levels of that tumor; between brackets are the number of cells in which the abnormality was found; * the mentioned chromosome is clonally over- or underrepresented in more than one copy; S, sex chromosome

Figure 2



G-banded karyotype of yolk sac tumor E86-893, arrows indicate aberrant chromosomes.

Figure 3



G-banded karyotype of yolk sac tumor E86-905, arrows indicate aberrant chromosomes.

actually meant an underrepresentation of the distal half of chromosome 4. Seven tumors missed either a whole copy or parts of chromosome 4. A clonal aberration of chromosome 13 was found in 2 different yolk sac tumors with, in both cases, the break in Band D (Band D, respectively D2).

Flow cytometry was performed on frozen tissue of primary ED tumors, 34 teratocarcinomas, and only 6 yolk sac tumors were available. Investigation of all karyotyped primary teratocarcinomas revealed that they all had a diploid DNA content. This is in agreement with the chromosomal findings with one exception (Tumor E86-426) in which karyotypically 2 of 6 cells were tetraploid.

The results of flow cytometry of the yolk sac tumors are ambiguous. Some of the primary yolk sac tumors (E86-874, E86-893 and E86-905), which were karyotypically polyploid, did not show aneuploidy in the flow pattern. Two yolk sac tumors (E86-580 and E86-875) had, according to the flow cytometric measurement, a cell population with a DNA content of more than 2N. Tumor E86-580 had a DNA index of about 1.32, which is equal to about 53 chromosomes, while all polyploid karyotypes had 87 or more chromosomes. For Tumor E86-875 and E86-615 the values of flow cytometry (DNA index = 1.86 respectively 1.0) and karyotype analysis were in the same range.

DISCUSSION

Chromosomal abnormalities are regularly associated with human and animal malignancies [see Heim and Mittelman, 1987; Sandberg *et al.*, 1988; Sasaki, 1982, for review]. A number of spontaneous and induced teratocarcinomas has been investigated cytogenetically. Most of them have a near diploid chromosomal complement. Some of the teratocarcinomas even were chromosomally normal [Cronmiller and Mintz, 1978; Mintz and Cronmiller, 1981; McBurney and Strutt, 1980], although they all were studied after *in vivo* and/or *in vitro* passaging. Data of 13 different aberrant teratocarcinomas (e.g.,

EC cell lines and their derivatives) reveal several common chromosomal abnormalities: loss of a sex chromosome; trisomy of chromosomes 6, 8 and 11; a deletion of chromosome 14; and an elongation of chromosome 1 [Martin *et al.*, 1978; Cronmiller and Mintz, 1978; McBurney, 1976; Iles and Evans, 1977; McBurney and Adamson, 1976; McBurney and Rogers, 1982; McBurney, 1989; Nicolas *et al.*, 1976]. All, except one, of the chromosomally abnormal teratocarcinomas had at least one of the above mentioned abnormalities.

In this paper we describe the cytogenetical findings of primary ED tumors. In total 34 of 36 teratocarcinomas, 3 of 18 teratomas and 9 of 27 yolk sac tumors could be karyotyped. Contrary to already published results, in this study the majority of the teratocarcinomas were chromosomally normal (82%). Flow cytometry on frozen tissue of primary teratocarcinomas confirmed the karyotypical findings: all had a diploid DNA content. This discrepancy with the literature is probably due to duration of culture time. We karyotyped the primary teratocarcinomas after a short culture time (less than 45 days) while, in the studies cited from the literature, karyotyping was performed on established EC cell lines. So it might be that the chromosomal abnormalities of malignant teratomas described in the literature are associated with *in vitro* karyotype evolution rather than with teratocarcinogenesis and/or tumorprogression. To investigate this possibility we now cytogenetically investigate retransplantation generations of our primary teratocarcinomas. It is of interest that 5 of 6 primary teratocarcinomas with a chromosomal aberration all originated in a female recipient. In 3 of them, one of the above-described common chromosomal abnormalities was found: loss of a Y chromosome in 2 tumors and a trisomy 8 in one tumor.

The cytogenetical findings of the teratomas and yolk sac tumors we karyotyped are in striking contrast to those of the teratocarcinomas. In total, 10 of 12 primary ED tumors without EC cells were chromosomally highly abnormal. All, except one, aberrant tumors were polyploid. To investigate

whether polyploidization occurred *in vivo* or *in vitro* flow cytometry was performed on frozen tissue of 6 yolk sac tumors. In 3, the flow cytometric results were in agreement with the karyotypical data, but in the other 3, flow cytometry revealed only a diploid cell population while the karyotypes showed polyploidy, so apparently here the polyploidization occurred *in vitro*. Some numerical abnormalities as well as structural abnormalities involving the same chromosome were found in different tumors. In 7 tumors one or more of the following chromosomes (Nos. 1, 6, 15, and 19) were overrepresented. Trisomies of chromosomes 6 and 15 have been described in teratocarcinomas [Martin *et al.*, 1978; Cronmiller and Mintz, 1978; McBurney, 1976; Iles and Evans, 1977; McBurney, 1989], lymphoid tumors, and myeloid leukemias [Wirschubsky *et al.*, 1984]. On both, chromosome homeoboxes as well as oncogenes, known to be important in normal proliferation and differentiation, are located [Buckle *et al.*, 1984; Rabin *et al.*, 1986]. Inappropriate expression of such genes might be important for tumorigenesis. In 7 tumors an underrepresentation of one or more of the following chromosomes (Nos. 2, 4, 14 and a sex chromosome) was found. Loss of a sex chromosome has been described in several animal tumor systems, and it is thought to be a secondary aberration [Sasaki, 1982; Dowjat and Wlodarska, 1981]. Partial deletion of chromosome 2 has been described for myeloid leukemia [Hayata *et al.*, 1983] and is associated with the genesis of such tumors in mice. Partial deletion of chromosome 14 has been described for teratocarcinomas [Iles and Evans, 1977; McBurney and Adamson, 1976; McBurney, 1989; Nicolas *et al.*, 1976]. Although the biological significance of this abnormality remains obscure, it seems to be restricted to germ cell tumors. The possible meaning of the loss of chromosome 4 will be discussed later. In 6 tumors an aberrant chromosome 1 was found in addition to the expected number of copies of chromosome 1. The common part of chromosome 1 that was overrepresented in the only 2 clonal cases is Band 1D to Band 1H. Probably the distal part of chromosome 1 contains genes important for tumor

progression and/or oncogenesis. In this respect it is of interest that the oncogene *bcl-2* has been mapped to chromosome 1, but the exact location is not known [Negrini *et al.*, 1987]. A study by Mock *et al.* [1988] revealed linkage of *bcl-2* to the *Idh-1/Pep-3* region of murine chromosome 1. This implies that *bcl-2* is located in the upper half of chromosome 1 [Buckle *et al.*, 1984]. An abnormal chromosome 4 was found in 5 different tumors, in all cases resulting in an underrepresentation of the distal part of chromosome 4 (Band 4E2 to 4qter). In another 2 tumors, whole copies of chromosome 4 were missing. These findings might suggest that a suppressor gene is located on the distal part of chromosome 4. Evans *et al.* [1982] found that suppression of malignancy in hybridoma cells was the result of either the loss of chromosome 4 from the normal fibroblasts or the gain from chromosome 4 of the tumor cells. They concluded that a gene on the normal chromosome 4 is responsible for the suppression of malignancy in a dose-dependent manner. These results are interesting, not only because they showed that different tumors may have a genetic lesion in common, but also because regulation in a dose-dependent manner corresponds with our results. A clonal aberration of chromosome 13 was found in 2 yolk sac tumors and, in both cases, the breakage had occurred in Band D.

To our knowledge this is the first time that primary teratomas and yolk sac tumors have been karyotyped, but there are some data on established cell lines of somatic derivatives. Iles and Evans [1977] observed a transformation to yolk sac carcinoma in embryoid bodies arising from an ED teratocarcinoma (Tumor 17). This transformation was accompanied by gross chromosomal abnormalities. Lehman *et al.* [1974] isolated 2 cell lines (PYS 1 and PYS 2) from an ED teratocarcinoma (original tumor was OTT6050) containing parietal yolk sac cells. They have been passaging these lines over 40 times during a 9-mo period, and both had a hypotetraploid chromosomal mode. Speers and Altman [1984] induced an embryonal carcinoma cell line (PCC4 AZA1) to differentiate *in vivo*

using retinoic acid. Two of the resulting differentiated tumors (one a chondrosarcoma and one a glioma/chondrosarcoma mixture) were retransplantable and chromosomally abnormal. The chondrosarcoma had a modal number of 66 chromosomes, whereas the glioma component of the other tumor had a mode of 37 chromosomes with 20% tetraploidy.

Our cytogenetic data support the concept that the oncogenesis of teratocarcinomas, which are considered malignant tumors, is developmental in origin, probably without a mutational event. The oncogenesis of yolk sac tumors, on the other hand, is mutational in origin in view of their cytogenetical aberrations and long latency.

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CHAPTER IV

ROLE OF THE SEX OF THE GRAFTED EMBRYO IN DETERMINING MALIGNANT DEVELOPMENT OF EMBRYO-DERIVED TUMORS

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Submitted

INTRODUCTION

Teratomas can be produced in most inbred strains of mice by grafting pre- or postimplantation embryos to the testes or under the kidney capsule of syngeneic recipients [Stevens, 1970a, 1970b; Damjanov *et al.*, 1971]. These embryo-derived (ED) tumors, which are indistinguishable from spontaneous teratomas, develop either benign or malignant. Benign teratomas, or simply teratomas, are composed of disorganized mature somatic tissues with a limited capacity of growth. Malignant teratomas, also known as teratocarcinomas, not only contain various mature and immature somatic tissues, but also undifferentiated embryonal carcinoma (EC) cells with capacity to proliferate. In addition ED tumors may develop along extra-embryonic lineage to form malignant yolk sac tumors [Martin, 1975; Van Berlo *et al.*, 1990].

A number of factors possibly regulating tumorigenesis in ED tumors has been investigated: gender of the host and strain-specific factors [Solter *et al.*, 1979], type of embryonic graft [Solter *et al.*, 1980], factors in F1 hybrid hosts [Solter *et al.*, 1981], immune factors [Damjanov *et al.*, 1982] and maternally transmitted factors [Damjanov and Solter, 1982]. From these studies it was evident that the tumorigenesis of ED tumors is influenced by both genetic and epigenetic factors. On the other hand the studies showed that the yield of teratocarcinomas can be modified by manipulating the embryo or the host. To explain the fact that in most mouse strains a 50% yield of teratocarcinomas is obtained from transplanted embryos, Damjanov *et al.* [1983] supposed that tumorigenesis is regulated by positive and negative stimuli and that it is only by chance that one of these factors will prevail.

The purpose of this study is to establish the relationship between the sex of the transplanted embryo and the possibly evolving malignancy of ED tumors.

MATERIALS AND METHODS

To produce tumors, embryo transplantations were carried out in inbred BALB/c mice (supplier: Harlan Olac, Woudenberg The Netherlands, formerly CPB, TNO Woudenberg). Seven day old embryos were transplanted underneath the kidney capsule of syngeneic male and female recipients. Representative samples of all primary ED tumors were taken for histology, tissue culture and retransplantation as described before [Van Berlo *et al.*, 1990]. In total 3 series of embryo transplantations were carried out. The three series were different with respect to the sex of the recipient animals. In the first series only female recipients were used, in the second series with few exeptions male recipients were used, and in the last series male and female recipients were used alternately.

The sex chromosomal constitution of primary and/or retransplanted ED tumors was determined by karyotype analysis, usually of 10 metaphases. Culture techniques and chromosome staining were carried out as previously described [Van Berlo *et al.*, in press]. The sex chromosomal constitution of the primary ED teratocarcinomas of the first series was also determined at the DNA level with a Y-specific probe. DNA was isolated from frozen tissue, cut into fragments by the restriction endonucleases Alu I and Hind III, blotted and hybridized with the male specific BKM-probe M13mp9 [Singh *et al.*, 1984].

The data were statistically evaluated with a loglinear model. 95% Confidence intervals were computed according to van der Waerden [1971].

RESULTS

In the course of this study 201 embryo transplantations were carried out. The majority of the grafted animals (170) developed a tumor. On histological criteria 59 primary tumors were classified as teratocarcinomas, 58 as teratomas,

and 53 as yolk sac tumors (table 1).

Most (57/59) teratocarcinomas could be karyotyped (table 2). Thirtyeight developed from an embryo with a sex chromosomal complement opposite to that of the recipient whereas 19 developed from an embryo with the same gender as the recipient. This difference is significant, 95% confidence interval $0.53 < p_{opposite} < 0.78$. In those cases where a teratocarcinoma had the same sex as the recipient, we either tried to retransplant the tumor into a recipient

Table 1: Histology of 201 embryotransplantations

	series I recipient XX	series II recipient XX	series II recipient XY	series III recipient XX	series III recipient XY	total recipient XX	total recipient XY	*total*
teratocarcinomas	17		19	12	11	29	30	*59*
teratomas	8	2	8	18	22	28	30	*58*
yolk sac tumors	11	1	14	13	14	25	28	*53*
take failures	4	1	12	9	5	14	17	*31*
total	*40*	*4*	*53*	*52*	*52*	*96*	*105*	*201*

of the opposite sex, and karyotype it again, or we investigated the primary tumor with a Y-specific probe. By the first method the sex of five female and nine male teratocarcinomas was confirmed. The sex chromosomal constitution of 15/29 primary ED teratocarcinomas, induced in a female recipient (the first series), was reinvestigated with a Y-specific probe. The probe gave no signal in the four karyotypically female tumors. Eight out of 11 teratocarcinomas proved to be Y-probe positive. The other three tumors gave no detectable signal, but

in these three cases karyotype analysis revealed a 40,XY/39,X,-Y mosaicism. Thus it might be that in these tumors the number of cells with a Y-chromosome was too small to allow detection with the probe. The results with the Y-probe therefore indirectly confirm the sex chromosomal constitution of the four karyotypically female tumors. The results of both methods combined, the sex chromosomal constitution of 8/10 female and 9/9 male teratocarcinomas was confirmed.

Teratomas were difficult to karyotype because these tumors grew poorly *in vivo* as well as *in vitro*. The difference between the number of teratomas with the same sex as the recipient and the number of teratomas with a sex chromosomal constitution opposite to that of the recipient is not significant (eight respectively ten).

Yolk sac tumors grew better than teratomas. In total 28 out of 53 yolk sac tumors could be karyotyped: a significant excess (23 out of 28) of tumors with the same sex as the recipient was found, 95% confidence interval $0.64 < p_{identical} < 0.92$. Since most yolk sac tumors proved to be retransplantable, we tried to karyotype the tumor again after retransplantation in a recipient of the opposite sex. Four tumors could be investigated by this method, all showing the opposite sex as compared to the primary tumor. Instead of tumor cells probably host cells were karyotyped. Thus this method is not reliable as a means to confirm the sex of the primary yolk sac tumors.

The experiments were conducted in three series, mostly identical in setup and procedures. In the first series only female recipients were used, while in the second series with few excetions male recipients were used. In the third series male and female recipients were used alternately. Separate analysis of the experiments showed identical trends, and there is no indication that time related factors have introduced recipient related artifacts. The data are therefore analyzed together. The first two hypotheses test the possibility of bias in the karyotyping procedure: male or female karyotypes may be easier to obtain, or karyotypes may be easier to obtain from male or female recipients.

Table 2: Sex chromosomal constitution of primary ED tumors

tumor/ recipient	teratocarcinomas series I	II	III	n	teratomas series I	II	III	n	yolk sac tumors series I	II	III	n
identical				*19*				*8*				*23*
female/female	4[1,2]		6[4]	10		1	2	3	3	1	5	9
male/male		6[3]	3[5]	9		1	4	5		6[6,7]	8[8]	14
opposite				*38*				*10*				*5*
male/female	11[1]		6	17			5	5			3[9]	3
female/male		13	8	21		1	4	5		2		2

[1] molecular data of 15 tumors: in 8 cases a signal was obtained with the Y-probe, the other 7 tumors gave no signal: 4 tumors were karyotypically female, the karyotypes of the 3 other tumors revealed a 40,XY/39,X,-Y mosaicism
[2] sex of 1 tumor was confirmed in retransplanted tumor in a male recipient
[3] sex of all 6 tumors was confirmed in retransplanted tumor in a female recipient
[4] sex of 4 tumors was confirmed in retransplanted tumor in a male recipient
[5] sex of all 3 tumors was confirmed in retransplanted tumor in a female recipient
[6] one tumor had only one sx chromosome, it is assumed that the Y-chromosome has been lost
[7] sex of 1 retransplanted tumor in a female recipient was female (with clonal abnormalities)
[8] sex of 2 retransplanted tumors in female recipients were female (1 with clonal abnormalities)
[9] sex of 1 retransplanted tumor in a female recipient was female (with clonal abnormalities)

Assuming that the sex of embryos is equally probable male or female, we expect the same number of male and female tumors. Almost exactly this proportion is observed (cytogenetically): 53 male and 50 female tumors. In total 105 male and 96 female recipients were used. Karyotypes were obtained from tumors in 56 male recipients and 47 female recipients, indicating that the sex of the recipient has no influence for obtaining a karyotype. Table 2 shows the

number of tumors found for each tumor type, depending on the sex of the grafted embryo and the sex of the recipient. It appears that two interacting factors are present, explaining the results. The first factor is that the ratio of male and female tumors depends on the type of the tumor. The second factor is that the data for male and female recipients appear identical when the classification variable "difference in sex between the tumor and the recipient" is used instead of "sex of the recipient". With these two classification variables a loglinear model (same or opposite sex x tumortype) gives excellent fit to the data, degrees of freedom=6, Pearson chi-square=2.26, p=0.89. An additive model (same or opposite sex + tumortype) gives a very poor fit, degrees of freedom=8, Pearson chi-square=21.00, p=0.007. Three teratocarcinomas, induced in female recipients, have a dubious classification, which implies that the 17/10 ratio may also be 14/13. The fit of the model then indeed slightly deminishes (Pearson chi-square=4.05, p=0.669), but is still quite good, an additive model gives a poor fit (Pearson chi-square=19.24, p=0.014). Thus one can conclude that the combination of the sex of the embryo with that of the recipient has significant influence on the evolving tumor type after embryo transplantation.

DISCUSSION

This study on the sex chromosomal constitution of ED tumors indicates that ED tumorigenesis in BALB/c mice might depend on the combination of the genders of embryo and recipient. Most teratocarcinomas had a sex chromosomal constitution opposite to that of the recipient (38/57), whereas most yolk sac tumors had the same sex chromosomal constitution as the recipient (21/26); for teratomas the differences are not significant (8 same/10 opposite).

The sex chromosomal constitution of primary ED tumors was determined by

karyotyping the primary and/or retransplanted tumors, and by using a male specific probe. Finding an opposite sex for a tumor as compared to the gender of the recipient clearly demonstrates that tumor cells were karyotyped. However, in those cases where the primary tumor had the same sex as the recipient, there is a possibility that instead of tumor cells host cells were karyotyped. To exclude this possibility two methods were applied: karyotype analysis after retransplantation in a recipient of the opposite sex, or molecular analysis of the primary tumor with a Y-specific probe. Karyotype analysis has the advantage over molecular analysis in that any chromosomal abnormalities may be found, and that it can be used for tumors induced in male and female recipients, whereas a Y-probe can only be reliably used for tumors induced in female recipients. Furthermore, if no signal is obtained with a Y-probe it does not necessarily mean that the investigated tumor is female, because of the possibility of the loss of a Y chromosome [Bunker 1964] from a male tumor and the occurrence of mosaic tumors with respect to sex chromosomes. Thus, to determine the sex chromosomal constitution of primary ED tumors, we recommend to karyotype the primary and/or retransplanted tumors. Since this method is not applicable to all ED tumors, we suggest to use molecular genetic techniques in addition. The polymerase chain reaction might be useful to determine the sex chromosomal complements of all primary tumors: male tumors in female recipients could be traced with a Y-probe, and female tumors in male and female recipients, as well as male tumors in male recipients could be traced by making use of RFLP polymorphisms of the X chromosome.

The cytogenetic data on teratocarcinomas indicate that karyotype analysis of retransplanted tumors is a reliable method for determining the sex of the primary tumor: in none of the investigated cases there were discrepancies between the sex of the primary tumor and the retransplanted tumor. In this respect the cytogenetic data of retransplanted yolk sac tumors are of interest. In four out of four cases the sex chromosomal constitution of the retransplanted tumor was opposite to that of the primary tumor, but the same

as that of the recipient. Thus probably host cells have been karyotyped. However, all four retransplanted tumors were aneuploid. Since the abnormal cells were near tetraploid, it is likely that they are the result of, *in vivo* and/or *in vitro*, polyploidization of host cells. Hybridization of two host cells is a possible mechanism. *In vivo* hybridization has been described: human-hamster [Goldenberg *et al.*, 1971, 1974], and mouse-mouse [Janzen *et al.*, 1971; Fenyö *et al.*, 1973]. In these systems tumor cells have hybridized with host cells or other tumor cells, whereas our results are more compatible with fusion of two host cells. Tveit *et al.* [1980] inoculated a human embryonal carcinoma in athymic mice. They found that culturing resulted in overgrowth of murine cells with chromosomal abnormalities. To explain their results they supposed that malignant transformation of mouse cells was induced *in vitro*, probably by human tumor cells at the start of the culture.

Our karyotypical data of the retransplanted yolk sac tumors could also be explained by an induction mechanism. Possibly polyploidization of host cells has occurred *in vivo* under the influence of chromosomally abnormal yolk sac tumor cells. The transformed host cells apparently have a growth advantage *in vitro*. It might be that polyploidization in general is stimulated by yolk sac tumor cells, for the DNA flow cytometry of a number of primary yolk sac tumors reveals a polyploid cell population (data not shown). It would be of interest to investigate the nature of the stimulus for polyploidization. From the above it is obvious that karyotyping retransplanted yolk sac tumors after culturing *in vitro* is not a reliable method to confirm the sex of the primary tumors. Therefore we are now reinvestigating the sex chromosomal constitution of primary and retransplanted yolk sac tumors with the polymerase chain reaction technique and a Y-specific probe. We use DNA isolated from cells collected prior to culture: frozen tumor tissue, collagenase suspension of tumor, and extra-embryonic tissue from the transplantated embryos.

Solter *et al.* [1979] investigated the role of strain specific factors and gender

of the recipient in ED tumorigenesis. They found that the incidence of teratocarcinomas varied from one strain to another, and that the sex of the recipient may influence this incidence in some strains, but not in others. For both male and female BALB/c recipients they found the same yield of teratocarcinomas. We too found no difference between the incidence of ED teratocarcinomas in male and female recipients. Although the sex of the recipient does not seem to influence the incidence of teratocarcinomas in BALB/c mice, Solter *et al.* [1979] observed a small difference between the tumor weights in male and female recipients. It is of interest to investigate whether different combinations of embryo/recipient genders give rise to differences in tumor weight.

The influence of immune factors in ED tumorigenesis has been studied by Damjanov and coworkers [1982]. They showed that mice depleted of X-ray and cyclophosphamide-sensitive cells can prevent ED teratocarcinogenesis. This effect can be overcome by the administration of thymic cells and partly by the administration of splenic cells of untreated animals. They hypothesized that either the thymus stimulates teratocarcinogenesis or, alternatively, various interacting components of the immune system protect the graft from effector cells (e.g. macrophages, natural killer cells). An other possibility is that cytokines of locally present lymphocytes stimulate tumor growth. Our results indicate that embryos with a sex chromosomal constitution opposite to that of the recipient have a greater chance to develop into a teratocarcinoma than embryos with the same sex as the recipient. It would be of interest to investigate whether and how a recipient is able to recognize an opposite sex chromosomal constitution of the transplanted embryo. Expression of male specific antigen (H-Y, for which the gene is located on the short arm of the Y-chromosome, Mclaren *et al.*, 1988) could play a role, and one would expect male recipients to be less able to recognize a female embryo than female recipients to recognize a male embryo. If this is true the latter combination might give rise to more and/or bigger teratocarcinomas. Our present study does

not answer this question.

Hormonal influences on ED tumorigenesis have only been studied in female recipients [Damjanov *et al.*, 1983]. In the teratocarcinoma permissive strain C3H significantly more embryonic grafts developed into a teratocarcinoma in pregnant recipients than in either virgin recipients or multiparous mice. These results indicate that the stimulatory effect could be due to placental hormones and/or growth factors, however it is also possible that the effect is related to immune changes due to pregnancy. Our results seem to indicate that the sex of the recipient by itself, and by inference sex hormones, have little influence on the outcome of embryo transplantation.

We recently studied the involvement of chromosomal abnormalities in ED tumorigenesis [Van Berlo *et al.*, in press]. We found that almost all (82%) primary teratocarcinomas were chromosomally normal, whereas almost all karyotyped yolk sac tumors had an abnormal chromosomal constitution (mostly polyploidy). It was hypothesized that the oncogenesis of teratocarcinomas is developmental in origin while the oncogenesis of yolk sac tumors is probably mutational in origin.

All above mentioned factors do have an influence on ED tumorigenesis, but their effect cannot be predicted. However the combination of the sex of the embryo and the recipient seems to have some predictive value. Opposite sex favors the development of teratocarcinomas, whereas the same sex of the graft and the recipient will give rise predominantly to yolk sac tumors. Apparently a recipient animal suppresses teratocarcinoma development more effectively when a grafted embryo is of its own sex, than when it is of the opposite sex. One is tempted to speculate that male recipient tissue more effectively induces differentiation ("regulates") of a male grafted embryo, and that female recipient tissue more succesfully regulates a female grafted embryo. There might be an analogy here with the regulation of EC cells by an embryo [Brinster, 1974]. At least one chimera has been produced with EC cells and an embryo of the opposite sex [Cronmiller and Mintz, 1978]. In this context it would be of

interest to know whether the combination of the sex of the injected EC cells and of the recipient embryo does influence quantitatively and qualitatively the production of chimeric animals and tissues. The observation that four cancers (teratocarcinoma, neuroblastoma, melanoma, and leukemia) can be regulated by their approriate embryonic fields, leads Pierce and Speers [1988] to postulate that there is an embryonic field capable of regulating tumor formation of every carcinoma. One could carry this speculation further by assuming that adult tissues may still have some capacity for regulation. In fact the clearest human example of effective regulation i.e. spontaneous regression and terminal differentiation of neuroblastoma IVs [Sitarz *et al.*, 1975], takes place in adult somatic tissue. The kidney may be particularly equipped to regulate pluripotent EC cells, as the blastema of the kidney itself is composed of pluripotent cells, which may give rise to nephroblastoma which apart from teratomas, is the most pluripotent of embryonal tumors.

Regulated grafts develop into mature teratomas. Karyotypically abnormal teratomas do not behave malignant, because they are composed of terminally differentiated cells. These neoplasms very much resemble residual mature teratomas after chemotherapy of disseminated nonseminomatous germ cell tumors of the testis, which are histologically fully differentiated, have low malignant potential, and are yet karyotypically highly abnormal [Oosterhuis *et al.*, 1986; Castedo *et al.*, 1989]. It is only in slowly growing murine teratomas that cells of extraembryonic (yolk sac) differentiation, which escape regulation by somatic tissues, are not overgrown, and enabled to acquire genomic aberrations leading to malignant transformation [Van Berlo *et al.*, 1990, in press]. Epidemiologic data on human germ cell tumors suggest that germ cell tumors with male genotypes are inherently more malignant than those with a female constitution. In contrast, our data indicate that in mice the sex of the tumor does not influence its malignancy. Extrapolating the mouse data to human pathology one would have to question the role of sex of the tumor as a

determinant of germ cell malignancy.

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CHAPTER V

SACRAL TERATOMA WITH LATE RECURRENCE OF YOLK SAC TUMOR: HUMAN COUNTERPART OF EMBRYO- OR YOLK SAC DERIVED TERATOMA?

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Submitted

ABSTRACT

A case is described of a baby boy who at birth had a large sacral teratoma without malignant components and a diploid DNA content (DNA-index = 1). A recurrence 19 months later was histologically a pure yolk sac tumor with two stem lines with a DNA-index of 1, and 1.79 respectively. Two karyotypes were highly abnormal with chromosome numbers corresponding with the aneuploid stem line. There was no isochromosome 12p marker present. After intensive chemotherapy a small residual mature teratoma (RMT) was excised with a DNA-index of 1.

The evolution of the tumor is remarkably similar to that of murine embryo-, and yolk sac-derived teratomas, in which after long follow up often yolk sac carcinoma develops. We propose that sacral teratomas rather than gonadal germ cell tumors are the human counterpart of murine embryo-, and yolk sac-derived teratomas.

INTRODUCTION

Sacral teratoma is the most frequent neonatal solid tumor, with an incidence of about 1 in 40.000. It has a strong predilection for girls, but a malignant course is more frequent in boys. The prognosis of the tumor is favorable when only mature and immature teratoma is found histologically. When other components are present, in particular yolk sac tumor, the tumor behaves as a malignant germ cell tumor. Early, radical surgery is recommended because late, incomplete surgery is associated with a high risk of malignancy, usually in the form of yolk sac tumor. The poorer prognosis of deep seated tumors may be due to later detection, and more difficult surgery [see Gonzales Crussi, 1982; and Damjanov *et al.*, (eds), 1983, for excellent reviews].

We describe a neonatal boy with a sacral teratoma, which 19 months after

surgery recurred as a yolk sac tumor. Following multiple drug chemotherapy a RMT was removed. DNA flow cytometry was done on all specimens, and karyotyping was done of the recurrence.

Case report

A neonatal boy presented with sacral mass which was clinically diagnosed as a conjoined twin. At operation, one day after birth, the lesion seemed not attached to surrounding structures, therefore, no need was felt to remove the coccyx. The surgical specimen measured 16 cm in largest dimension. A foot-like structure protruded from the tumor, which was entirely covered by skin (Fig. 1). A specimen X-ray showed many bones, some of them clearly recognizable as bones of malformed extremities, in particular within the "foot" (Fig. 2). However, there was no axialization and metameric segmentation in the lesion. Microscopic examination revealed many different somatic tissues, all of them with a high level of organoid differentiation (Fig. 3). Components other than mature teratoma were not found. The surgical margins were narrow, but the resection seemed radical. Nevertheless, 19 months later a local recurrence was noted. Serum levels of alfa-fetoprotein were now for the first time elevated. A biopsy showed only yolk sac tumor (Fig. 4). After platinum based, multiple drug chemotherapy a residual tumor mass was resected, which was solely composed of mature teratoma (Fig. 5). Presently, 15 months after diagnosis of the recurrence, the infant has no evidence of disease.

MATERIALS AND METHODS

The primary tumor was submitted in formalin, making tissue culture impossible. The biopsy of the relapse and the small residual tumor were submitted fresh and sterile. Tumor tissue was processed for tissue culture,

karyotyping and DNA flow cytometry as described [Oosterhuis *et al.*, 1986].

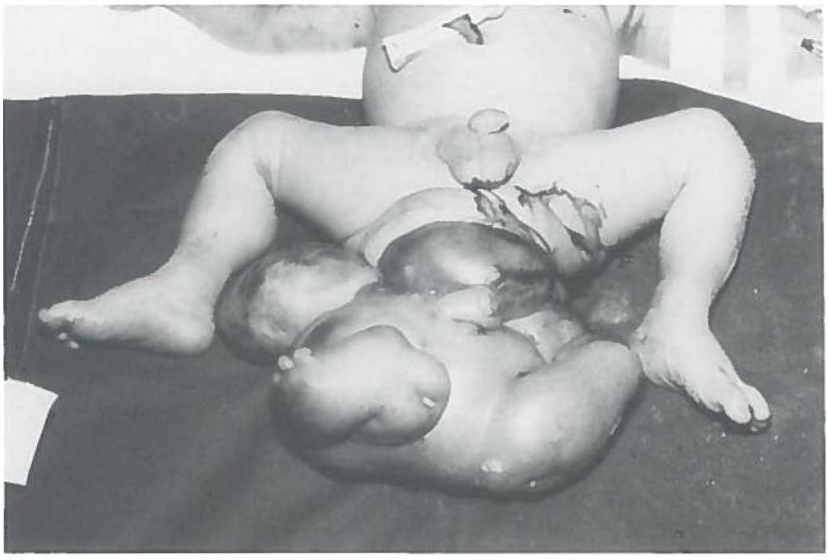
Tumor ploidy was expressed by the DNA index (DI) defined as the ratio between the modal G0,1 peak of the aneuploid population to that of the modal G0,1 peak of diploid normal cells in the samples. By definition a diploid tumor cell population thus has a DI = 1.00. In the DNA profiles the most left peak was considered to represent the diploid population. A tumor was classified as aneuploid when the DNA profile showed two distinct G0,1 peaks and as multiploid when multiple aneuploid stem lines were present. When the DNA profile showed a shoulder, a single G0,1 peak with a high coefficient of variation (CV) or an ill-defined subpopulation the measurements were repeated. Tumors showing a single G0,1 peak with 5.5% < CV < 10% were classified as near-diploid. DNA profiles with CV's over 10% were considered uninterpretable.

RESULTS

The primary tumor, measured on paraffin embedded tissue, was diploid: DI = 1. The yolk sac tumor in the relapse had two stem lines in two separate samples: one diploid, and one aneuploid with a DI = 1.79. The RMT was diploid.

Due to a very low yield of mitoses in the yolk sac tumor, only two metaphases of substandard quality could be analyzed. They had several structural abnormalities in common. Fig. 6 shows one of the karyotypes with the description. The RMT was too small to allow tissue culture.

Figure 1

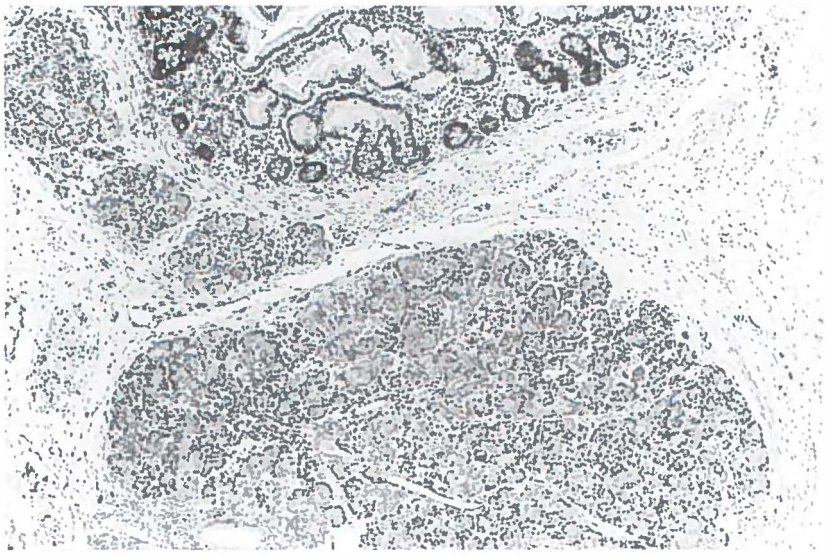


Sacral teratoma with a structure resembling a foot protruding in the middle. This tumor was resected, but recurred 19 months later as a pure yolk sac tumor.

Figure 2

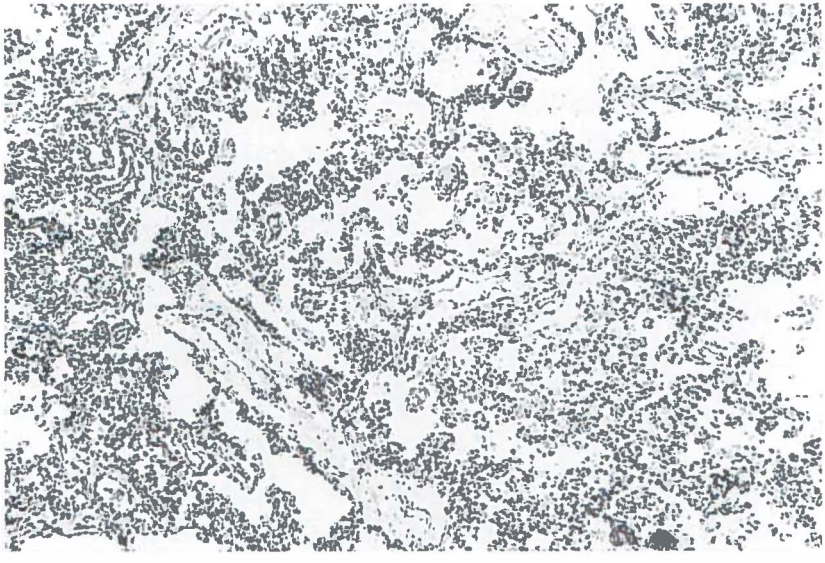
X-ray of the tumor shown in figure 1, There are many bones, some of them clearly recognizable as the skeleton of an extremity.

Figure 3



Histologically there was a high level of somatic differentiation in the sacral teratoma. Shown are tissues resembling pancreas and gut. No components other than mature teratoma were found (hematoxylin and eosin, x 56).

Figure 4



Recurrence of the sacral teratoma of figure 1 with the histology of a pure yolk sac tumor (hematoxylin and eosin , x 56).

Figure 5

Residual mature teratoma after chemotherapy of the recurrence. The wall of a cyst lined by ciliated epithelium is shown (hematoxylin and eosin, x 56).

DISCUSSION

In view of the absence of axialization and metameric segmentation, and the histology of mature somatic tissues, the lesion was pathologically classified as a mature teratoma and not as a conjoined twin [Gonzales Crussi, 1982].

The clinical course of the disease was classical in the sense that after incomplete surgery (the coccyx was not removed en bloc with the tumor) thetumor relapsed with a yolk sac tumor component [Pantoja and Roderiguez-Ibañez, 1976]. The residual mass after chemotherapy had the histology of mature teratoma. RMT is a common phenomenon after chemotherapy of disseminated nonseminomatous germ cell tumors of the testis with a teratoma component [Oosterhuis *et al.*, 1983]. In this case comparison of the DI of the primary tumor, the yolk sac tumor in the relapse, and the RMT strongly

Figure 6

Representative karyotype of the recurring tumor. Structurally abnormal chromosomes are indicated by arrows. The chromosome number of 83 is in agreement with the DI of 1.79. Isochromosome 12p, a marker, which is found in 80% of testicular germ cell tumors of adults, and in certain other germ cell tumors, is lacking.

83, XY, +X, +del(1)(p11), +2, +der(3), +der(3), +4, +5, +5, +der(6), +del(6)(q21), +der(7), +8, +8, +9, +9, +10, +12, +12, +13, +13, +15, +15, +16, +17, +18, +20, +21, +22, +22, +8 mar

supports the view that RMT is the result of selective destruction of components other than teratoma [Oosterhuis *et al.*, 1983].

The diploid nuclear DNA content of the primary tumor and the RMT is in keeping with the scarce published data on the ploidy of sacral teratomas [Kaplan *et al.*, 1979; Linder *et al.*, 1975]. The finding of a diploid and an aneuploid stem line in the yolk sac tumor suggests that this component has developed as a result of tumor progression. To our knowledge no karyotypes have been published of yolk sac tumor arising in sacral teratoma. In this case the isochromosome 12p is lacking. This is in keeping with our hypothesis that i(12p) is related with germ cell tumors which are either gonocytomas (seminoma, dysgerminoma, and germinoma) or have developed through a gonocytoma stage [Damjanov and Oosterhuis, paper in preparation]. Such "true" germ cell tumors occur in anatomical localizations where gonocytomas are found: the gonads, the anterior mediastinum, and the midline of the brain. Isochromosome 12p was demonstrated in about 80% of testicular germ cell tumors, regardless of histology [Castedo *et al.*, 1989a, 1989b], in two dysgerminomas of the ovary [Atkin and Baker, 1987; Jenkyn and McCartney, 1987], and in at least 3 mediastinal malignant germ cell tumors [Damjanov and Oosterhuis, paper in preparation; Dal Chin and Van den Berge, paper in preparation; Chaganti *et al.*, 1989]. Very recently it was demonstrated in a nonseminomatous germ cell tumor of the pineal gland [R.M. Slater, personal communication]. Teratomas in other anatomical localizations, in particular sacral teratomas never contain a gonocytoma-component [Gonzales Crussi, 1982]. They are probably not derived from germ cell precursors, but from pluripotent embryonal, or extra-embryonal stem cells, which have escaped organization [Gonzales Crussi, 1982]. These pluripotent cells may produce neoplastic embryonal, and extra-embryonal tissues, but apparently never tumors corresponding to the germ cell lineage.

A comparison of murine embryo-derived teratomas and sacral teratomas

shows striking similarities with regard to cells of origin and biologic behaviour. Seven day old mouse embryos transplanted under the kidney capsule of syngeneic mice, followed for a period of eight weeks, develop into either benign teratomas or teratocarcinomas. Embryo-derived tumors develop from pluripotent embryonal cells, and not from germ cells present in the embryo [see Damjanov and Solter, 1974, for review]. The proportion of benign and malignant tumors depends on embryonic and host related factors [Solter *et al.*, 1979, 1980, 1981; Damjanov *et al.*, 1982, 1983]. Recently we have shown that after a long period of follow up (up to one year), more than 50% of the teratomas develop a yolk sac tumor component. A considerable proportion of the tumors had a malignant phenotype, and proved retransplantable. Thus, embryo-transplantation may result in a teratoma which after long follow up develops into a yolk sac carcinoma [Van Berlo *et al.*, 1990].

A similar course of events is found in fetectomy experiments in mice and rats, also an animal model for teratomas [Sobis *et al.*, 1982, 1983]. First, after a follow up of several weeks teratomas develop, and only after long follow up in the order of months, do yolk sac tumors appear. In this model system it was also convincingly shown that the tumors do not originate from germ cells, but from pluripotent stem cells either already present in the endoderm of the visceral yolk sac, or newly appeared as a result of dedifferentiation of extra-embryonal cells [Sobis and VandePutte, 1975]. Again the scenario is that yolk sac carcinoma develops from teratoma after a long latency.

We propose that sacral teratoma, which as in the case described here, may recur as yolk sac tumor, is the human counterpart of the embryo- or yolk sac derived teratoma in mice and rats. In this context it is relevant that the original teratoma was diploid, but the late recurring yolk sac tumor had an aneuploid stem line in addition to a diploid one. We have similarly found that early appearing murine embryo-derived teratoid tumors are diploid, but that the late appearing yolk sac tumors are often aneuploid, in agreement with their

malignant phenotype [Van Berlo *et al.*, in press].

Accepting the conclusion that murine embryo- and yolk sac derived tumors are models for sacral teratomas in particular, and perhaps more in general for extragonadal teratomas (except those of the anterior mediastinum and the midline of the brain), implies that they are less relevant as models of gonadal germ cell tumors, in particular testicular ones. Gonadal germ cell tumors have in common, as opposed to embryo-derived tumors, that they are indeed, albeit with different mechanisms, derived from germ cell precursors. This is probably also true for germ cell tumors of the anterior mediastinum and the midline of the brain [Friedman, 1987]. Another obvious difference between the murine models and the germ cell tumors of the gonads, the anterior mediastinum and the midline of the brain, is that the former never have a gonocytoma component.

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CHAPTER VI

GENERAL DISCUSSION

The main goal of the work presented in this thesis was to examine whether murine embryo-derived (ED) teratomas could be used as models for human teratomas.

Murine ED teratomas are not derived from germ cells, but from embryonal cells [Mintz *et al.*, 1978]. A human counterpart of this murine model is therefore not to be found in the gonads as teratomas in the gonads are derived from germ cell progenitors [Damjanov, 1990]. But even among the extragonadal teratomas some may be derived from germ cell precursors. This is probably true for the extragonadal teratomas in the anterior mediastinum and the midline of the brain. An argument for this assumption is the histological composition of teratomas in these anatomical localizations: they may be partly or entirely be composed of neoplastic gonocytes (early germ cell precursors) [Gonzales Crussi, 1982]. Another argument is the finding that teratomas of the anterior mediastinum and the midline of the brain may have the isochromosome 12p marker, which is characteristically found in the gonadal gonocytomas and in gonadal nonseminomatous germ cell tumors which have evolved through a gonocytoma stage [Oosterhuis *et al.*, 1989; De Jong *et al.*, 1990, in press; Oosterhuis *et al.*, 1990, in press]. Extragonadal teratomas other than in the anterior mediastinum and the midline of the brain consistently lack a gonocytoma component. Moreover, isochromosome 12p was not demonstrated in these tumors. It must be stated however, that cytogenetic data on these teratomas are scarce.

The most common of the "non-germ derived" extragonadal teratomas is the sacral teratoma of infants. These tumors are in our view the real human counterpart of murine ED teratomas. This view is further supported by our finding, reported in chapter II, that ED teratomas may after long latency progress to form retransplantable cytogenetically abnormal yolk sac carcinomas. The similarity with the case of a sacral teratoma recurring as a yolk sac tumor in an infant boy described in chapter V is striking indeed. In this case the original sacral teratoma was diploid similar to the early ED teratocarcinomas

and teratomas (chapter III). On the other hand, the recurring yolk sac tumor was aneuploid and karyotypically abnormal in keeping with many of the late appering ED yolk sac carcinomas (chapter III).

In humans, teratomas are much more frequent in females than in males, but malignant teratomas are most often found in males. This applies also to sacral teratomas. The overrepresentation of malignant teratomas in males could be due to an inherently greater chance for malignant behaviour of embryonal carcinoma (EC) cells with a male sex chromosomal constitution. The ED teratoma model is the ideal system to test this hypothesis, as it is possible to graft male and female embryos in recipients of either sex. Studies on the sex chromosomes of ED tumors (teratocarcinomas, teratomas and yolk sac carcinomas) have shown that the development of a teratocarcinoma is favored when the sex of the grafted embryo and the recipient is opposite (chapter IV). When the sex of the graft and the recipient is the same, the chances are that the EC cells in the tumor are regulated to form terminally differentiated mature teratoma. Cells of extraembryonic differentiation, in particular parietal yolk sac differentiation, seem to escape regulation, even when they are of the same sex as the recipient, and may after long latency accumulate genomic aberrations leading to malignant transformation. (A similar course of events may be postulated for the development of yolk sac tumor in sacral teratomas.) The results of the study on the role of the sex of the grafted embryo in its tumorous development clearly show that a male sex chromosomal constitution has no inherent malignant potential. Therefore, it is unlikely that the sex chromosomes account for the epidemiologically observed predominance of malignant teratomas in male patients. We conclude that:

1. Murine ED teratomas may progress into yolk sac carcinoma, a phenomenon also observed in human sacral teratomas.

2. The cytogenetic findings in murine ED tumors do not parallel those in human gonocytomas and nonseminomatous germ cell tumors evolved through a gonocytoma stage.

3. The cytogenetic findings in murine ED tumors are in keeping with the scarce cytogenetical data on human non-germ cell derived extragonadal teratomas exemplified by sacral teratomas.

4. Analysis of sex chromosomes of murine ED tumors demonstrates that neither a male nor a female sex chromosomal constitution of a grafted embryo has an inherent malignant potential.

5. Sacral teratomas are the human counterparts of murine ED teratomas.

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SUMMARY AND CONCLUSIONS

In this thesis two factors possibly regulating the malignant development of murine embryo-derived (ED) tumors have been investigated: the role of chromosomal abnormalities, and the possible role of the sex of the grafted embryo. To produce tumors, 7 day old BALB/c embryos were retransplanted under the kidney capsule of syngeneic male and female recipients. The mice were killed when the tumor reached a size of about 1.5-2.5 cm, or arbitrarily in the period of 225-365 days after embryo transplantation. Representative samples of primary ED tumors were taken for retransplantation, histology and tissue culture in order to analyze the chromosomal constitution of the tumors.

In chapter II the histological and biological characteristics of ED tumors are described. It is of interest that besides the expected teratocarcinomas and teratomas another tumortype evolved: yolk sac tumors. The yolk sac tumors developed after long latency. Moreover some of the larger yolk sac tumors proved to be retransplantable, and thus they have to be considered malignant. In the literature murine yolk sac tumors are described as a result of retransplantation of teratocarcinomas. However, we found yolk sac tumors as a result of embryo transplantation. Thus we showed that yolk sac tumors can reproducibly produced by embryo transplantation.

In chapter III the cytogenetics of primary ED tumors are described. Most (82%) primary teratocarcinomas had a normal chromosomal constitution, whereas almost all (7/9) karyotyped yolk sac tumors were chromosomally abnormal. The few abnormal teratocarcinomas showed different chromosomal abnormalities: loss of a Y chromosome, an elongated chromosome 5, trisomy 8, trisomy 19, and polyploidy. On the other hand most chromosomally aberrant yolk sac tumors had polyploidy in common. This finding is of interest since it is known that polyploidization as well as structural chromosomal abnormalities in particular isochromosome 12p probably play a role in the pathogenesis of human germ cell tumors.

In chapter IV the possible role of the sex of the grafted embryo in the malignant development of ED tumors is described. Most teratocarcinomas had

a sex chromosomal constitution opposite to that of the recipient, whereas most yolk sac tumors had the same sex chromosomal constitution as the recipient, for teratomas the differences are not significant. Thus we assume that the combination of gender of embryo and recipient does influence the outcome of embryo transplantation. The sex of the grafted embryo or of the recipient per se do not affect the result of embryo transplantation.

In chapter V a case report of a human sacral teratoma is described. Sacral teratomas occurre during neonatal life and are more frequent in girls than in boys. However, a malignant course is more frequent in boys. In this case the primary tumor recurred with the histology of yolk sac tumor. The recurrence was karyotyped and showed polyploidy. Flow cytometric measurement was done on the primary tumor, the recurrence and the mature rest lesion. The flow pattern of the recurrence showed a diploid and an aneuploid cell population (DI of the aneuploid stem line = 1.79). The primary tumor and the mature rest lesion had a DI = 1.00. The resemblance between the pathobiology of this case and that of the murine yolk sac tumors is remarkable.

In chapter VI the results are discussed and the following conclusions are drawn:

1. Murine ED teratomas may progress into yolk sac carcinoma.

2. The cytogenetic data on murine ED tumors differ from those of human germ cell derived teratomas.

3. The cytogenetic data on murine ED tumors are similar to those of human non-germ cell derived extragonadal teratomas.

4. The sex chromosomal constitution of a grafted embryo has no influence on the malignant development of murine ED tumors.

5. Sacral teratomas are the human counterparts of murine ED teratomas.

SAMENVATTING EN CONCLUSIES

In dit proefschrift zijn twee factoren onderzocht die mogelijk van invloed zijn op de maligne ontwikkeling van zogenaamde "embryo-derived" (ED) tumoren in de muis: de rol van chromosomale afwijkingen, en de rol van het geslacht van het getransplanteerde embryo. Om tumoren te produceren, werden zeven dagen oude BALB/c embryo's onder het nierkapsel van syngene mannelijke en vrouwelijke recipiënten getransplanteerd. De muizen werden gedood wanneer de tumor 1,5 à 2,5 cm groot was, of in de periode van 225 tot 365 dagen na de transplantatie van het embryo. Van de primaire ED tumoren werden representatieve delen gebruikt voor retransplantatie, histologie, en weefselkweek voor de analyse van de chromosomale constitutie van de tumoren.

In hoofdstuk II worden de histologische en biologische karakteristieken van de ED tumoren beschreven. Het is interessant dat naast de verwachte teratocarcinomen en teratomen nog een ander tumortype ontstond: dooierzaktumoren. De dooierzaktumoren ontstonden na een lange latentietijd. De wat grotere dooierzaktumoren bleken retransplantabel en moeten dus als maligne worden beschouwd. We hebben aangetoond dat de dooierzaktumoren reproduceerbaar geïnduceerd kunnen worden door middel van embryotransplantatie.

In hoofdstuk III wordt de cytogenetica van de primaire ED tumoren beschreven. Het overgrote deel van de teratocarcinomen (82%) bleek een normale chromosomale constitutie te hebben, terwijl bijna alle gekaryotypeerde (7/9) dooierzaktumoren chromosomaal afwijkend waren. In het geringe aantal teratocarcinomen met een abnormale chromosomale constitutie werden de volgende afwijkingen aangetroffen: het verlies van een Y chromosoom, een verlengd chromosoom 5, trisomie van chromosoom 8, trisomie van chromosoom 19, en polyploidie. Praktisch alle chromosomaal afwijkende dooierzaktumoren waren polyploid. De cytogenetische kenmerken van ED tumoren stemmen niet overeen met die van gonadale kiemceltumoren bij de mens, maar zijn meer in overeenstemming met de cytogenetische afwijkingen bij bepaalde extragonadale

teratomen.

In hoofdstuk IV wordt de mogelijke invloed van het geslacht van het getransplanteerde embryo op de maligne ontwikkeling van ED tumoren beschreven. Verreweg de meeste teratocarcinomen bleken een aan de recipiënt tegengestelde geslachtschromosomale constitutie te bezitten, terwijl de meeste dooierzaktumoren juist hetzelfde geslacht hadden als de recipiënt, voor teratomen waren dergelijke verschillen niet significant. Op grond van deze gegevens nemen wij aan dat de combinatie van het geslacht van het embryo en dat van de recipiënt van invloed is op het tumortype dat zich zal ontwikkelen na embryotransplantatie. Het geslacht van het embryo alleen, heeft geen invloed op het tumortype dat zal ontstaan na embryotransplantatie.

In hoofdstuk V wordt een pasgeboren jongetje met een sacraal teratoom beschreven. Sacrale teratomen worden vooral gezien bij pasgeboren zuigelingen en met name bij meisjes. Deze tumor kan maligne ontaarden en dan juist in de vorm van een dooierzaktumor. Maligne ontaarding doet zich vaker voor bij jongens. Ook in dit geval recidiveerde het sacrale teratoom als dooierzaktumor. Het recidief werd gekaryotypeerd en bleek polyploid, terwijl de primaire tumor diploid was. Er is een opvallende overeenkomst tussen de ED tumoren en het sacrale teratoom met betrekking tot evolutie en ploidie. Op grond hiervan concluderen wij dat het sacrale teratoom de menselijke tegenhanger is van het ED tumor model. Mee op grond van deze resultaten moet worden aangenomen dat sacrale teratomen niet uit kiemcellen maar uit pluripotente embryonale cellen ontstaan.

In hoofdstuk VI worden de resultaten bediscussieerd en de volgende conclusies getrokken:

1. ED teratomen in de muis kunnen uitgroeien tot een dooierzaktumor.

2. De cytogenetische gegevens van de ED tumoren in de muis komen niet overeen met de cytogenetische gegevens van die humane teratomen die uit kiemcellen zijn ontstaan.

3. De cytogenetische gegevens van de ED tumoren in de muis zijn

vergelijkbaar met de cytogenetische gegevens van die humane extragonadale teratomen die niet uit kiemcellen zijn ontstaan.

4. Het geslacht van het getransplanteerde embryo is niet van invloed op de maligne ontwikkeling van ED tumoren in de muis.

5. Sacrale teratomen vormen de humane tegenhanger van ED teratomen in de muis.