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REVIEW

Risk of tumorigenicity in mesenchymal stromal cell–based therapies—Bridging scientific observations and regulatory viewpoints

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Abstract

In the past decade, the therapeutic value of mesenchymal stromal cells (MSCs) has been studied in various indications, thereby taking advantage of their immunosuppressive properties. Easy procurement from bone marrow, adipose tissue or other sources and conventional *in vitro* expansion culture have made their clinical use attractive. Bridging the gap between current scientific knowledge and regulatory prospects on the transformation potential and possible tumorigenicity of MSCs, the Cell Products Working Party and the Committee for Advanced Therapies organized a meeting with leading European experts in the field of MSCs. This meeting elucidated the risk of potential tumorigenicity related to MSC-based therapies from two angles: the scientific perspective and the regulatory point of view. The conclusions of this meeting, including the current regulatory thinking on quality, nonclinical and clinical aspects for MSCs, are presented in this review, leading to a clearer way forward for the development of such products.

Key Words: *cell, mesenchymal, stromal, tumorigenicity*

Introduction

Mesenchymal stem cells/mesenchymal stromal cells (MSCs) have been studied and used for more than a decade now to treat various diseases (1). Human MSCs are most commonly isolated from the mononuclear fraction of the bone marrow (BM) or from adipose tissue. Other sources for MSCs are also used, for example, some cell preparations are isolated from placenta, amniotic fluid or periosteum (2,3). The isolated MSCs show phenotypic heterogeneity, depending on the origin of the cells and the isolation/manufacturing techniques. According to current

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thinking, the cell surface proteins expressed by MSCs include cluster of differentiation (CD)105, CD73, CD44, CD90, CD71, Stro-1, CD106 CD166 and CD29. MSCs do not express hematopoietic markers such as CD45, CD14 or CD11. They also do not express co-stimulatory molecules CD80, CD86 or CD40 or adhesion molecules CD31, CD18 or CD56. In 2006, the research groups within the International Society for Stem Cells agreed on the minimal cell surface markers, considered necessary to distinguish mesenchymal stromal cells from other cell types (4).

Several researchers have described a role of MSCs in tumor formation (5,6). Furthermore, some groups have reported that human MSCs may develop genetic instabilities and undergo a transformation process after long-term culture, as suggested for adipose-derived MSCs (7), and for BM-derived MSCs (8). In contrast, however, several authors have reported that MSCs cultivated *in vitro* can be expanded over multiple cell doublings without apparent loss of differentiation potential or chromosomal alterations (9,10). In addition, long-term cultured MSCs can develop chromosomal abnormalities but without evidence of transformation potential (11). This discrepancy may be explained by reports that the cell cultures used in the studies performed by Rubio *et al.* (7) and Rosland *et al.* (8) were contaminated by malignant cells that initially grew slowly in the presence of human MSCs (12,13).

In October 2011, the Cell Products Working Party (CPWP) arranged an expert meeting to discuss the findings of the research groups working in this area. The expert meeting was intended to obtain information and discuss the opportunities and challenges currently faced when MSCs are used as therapeutic products. The focus was on tumorigenicity as a safety concern frequently expressed by regulatory authorities, although practicalities involved in the development of MSCs as medicinal product were also more broadly discussed.

Manufacturing and quality aspects

Garcia *et al.* (12) and Torsvik *et al.* (13) reported that the original observations of tumor formation for isolated MSCs were the result of MSC cultures contaminated with tumor cell lines. The meeting experts therefore emphasized the importance of recommending that cell culture is performed under good manufacturing practice conditions to ensure proper segregation and control of starting and raw materials.

The discussions also highlighted some specific issues around terminology and the need for harmonization. For example, the time for cells in culture is often described in terms of "cell passages." This was considered a nonspecific term that does not permit a suitable comparison for standardization purposes. Thus, the use of "population doubling level" (PDL) was unanimously recommended both by the experts and CPWP members. Concerning the impact of varying PDLs, it would be of interest to compare the different cell culture protocols used by different developers of a cell-based product for same indication (e.g., graft versus host disease [GvHD]). Overall, the maximum PDL for cell culture processes must be justified at the time of marketing authorization application.

The expert group discussed whether the manufacturing process could trigger the generation of cytogenetic abnormalities and what risk factors could promote tumorigenicity in MSCs. During the discussion, it appeared that culture conditions and duration of the cell propagation significantly influence the formation of cytogenetic abnormalities. Most experts considered that long-term cell expansion of MSCs may increase the risk of chromosomal aberrations. Some experts, however, did report that no such phenomenon had been seen when their own cell culture processes were used.

It was concluded that such abnormalities can be avoided through the use of methods that ensure slow growth and short expansion times because the number of expanded cells is linked to the growth rate, and a high proliferative rate may potentiate risk of karyotypic changes. A low PDL number was also considered an important factor in this regard.

It was also hypothesized that physiological stress or *in vitro* culture conditions may significantly contribute to the occurrence of cell or chromosomal aberrations: for example, enzymatic cell dissociation (i.e., trypsin) raises more concerns in relation to abnormalities than mechanical dissociation. Several reports indicate that culture conditions could also affect chromosomal stability (14,15). Therefore, it is important to identify and define culture conditions during process development, which avoid the occurrence of chromosomal abnormalities.

Cells with chromosomal aberrations are known to be less able to divide, which may lead to apoptosis and death of the abnormal cells during the culture (16). It appears that the majority of abnormalities lead to senescence, but it is difficult to formally exclude the risk of cell transformation because deoxyribonucleic acid (DNA) damage is considered a central process in tumor formation. It has been suggested by some experts that cell senescence and transformation could be evaluated with the use of certain molecular markers (11). However, this issue was not discussed in the meeting, and the original results remain to be confirmed. It is therefore important, for each defined processes, to force a sample of cells on an experimental basis into senescence through the use of

long-term culture and to characterize the genetic and proliferative stability of the cells to justify the optimal culture time for the intended use.

The majority of experience reported during this meeting highlighted that the occurrence of genetic aberrations appears to be related mainly to the manufacturing process rather than to the patient-derived factors. However, there appeared to be divergent views on this aspect. Different manufacturing processes and laboratory practices between the research groups were identified. Various chromosomal abnormalities are sometimes seen from one patient to another. Further studies to investigate the role of the donor are therefore needed.

Cells with chromosomal aberrations

Observed chromosome aberrations can be broadly divided into two categories: (i) spontaneous abnormalities and (ii) recurrent abnormalities. Spontaneous (nonrecurrent) abnormalities are heterogeneous from one batch to another and from one patient to another. To exclude products containing cells with abnormalities potentially conferring a proliferative advantage, it was suggested that a karyotyping analysis would be sufficient as a release test in the case that it is possible (frozen product). A limit was suggested to be set with exclusion of two identical abnormal metaphases on 20 metaphases analyzed (10%). The International System for Human Cytogenetic Nomenclature in its latest Edition (2009) establishes that the same chromosome aberration must be found in at least two different cells to be considered as a clonal chromosome change. Recurrent abnormalities imply that the observed abnormalities are detected at least in two expanded cell samples originating from the same patient. In this case, more attention should be paid to potential risks, including testing at release. An assessment of the benefit-risk ratio linked to the clinical indication should be conducted before use of such cells.

If no chromosomal abnormalities are observed, there should be no need to perform cytogenetic testing on each batch as release criteria. In either case, it is important to cryopreserve cell samples at critical steps of manufacturing to test them later on, if needed.

During the discussion, it was reported that chromosomal abnormalities are rarely observed in primary cells (17). MSCs are adult stromal cells with a limited possibility of expansion in culture, contrary to embryonic stromal cells or induced pluripotent stromal cells. However, MSCs are a heterogeneous cell population composed of cells with different population doublings. Thus, some of the cells may be more prone to develop chromosomal aberrations. It should be emphasized that tumor formation in human patients after MSC administration has not been reported to date.

Other groups suggested the ability of MSCs to develop chromosomal aberrations in culture (18,19). The novelty of this field of research as well as the scarcity of clinical long-term follow-up may lead to different perspectives on these issues (20,21). Also, because of uncertainties on the characteristics of these abnormal cells and because of a limited follow-up of the patients, it is currently difficult to estimate the real risk these abnormalities may pose to patients.

Even if it cannot be demonstrated that the transformed cells do not lead to tumor formation, a possibility for such a risk remains to be considered. From an epidemiological point of view, there are not enough data available to make firm conclusions. To address this risk, it would be important to set up follow-up studies and/or registries to collect long-term data from treated patients.

Analytical techniques available to assess cytogenetic abnormalities

Two groups of techniques were discussed as possible methods for testing of genetic instability: (i) conventional karyotyping (GTG-banding, G bands by trypsin with the use of Giemsa) and Spectral karyotyping (SKY) technique) and (ii) molecular cytogenetic techniques (fluorescent *in situ* hybridization [FISH] and comparative genomic hybridization array (CGH array) and single nucleotide polymorphism array (SNP array). Karyotyping should be used to screen cells for chromosomal abnormalities during the preclinical phase of product development. At least 20 mitoses from several different production batches should be studied during the preclinical phase. It should be noted that conventional karyotyping has poor sensitivity and resolution (approximately 5−10 Mb resolution), which alone is not sufficient to predict full genetic stability. However, the assay is relatively easy to perform, and it allows detection of balanced rearrangements. In contrast, CGH array has higher sensitivity ($\leq$50 kb), although it may not be sufficient to detect a low proportion of cells with abnormalities (low mosaicism) and balanced rearrangements. Thus, karyotyping and CGH array should be used together as complementary tools. Characterization of putative chromosomal aberrations could be continued with the use of immunofluorescence and FISH, for example, to have further proof of genotypic stability. These techniques are able to detect minor structural abnormalities. FISH could be used to further investigate aberrations previously detected by means of karyotyping.

One of the difficulties for valid karyotype testing appears to be suitable positive controls (MSC transformed cells) for robust testing. Thus far, no

transformed MSCs but only immortalized MSCs (22) have been used as positive control. Furthermore, the status of the quality control laboratories, technical references (international guideline, Pharmacopoeia monograph or equivalent) and relevant tests to be performed need consideration. Currently, it appears that most karyotype tests are conducted for/within hospitals, and there are certain accreditations that a testing laboratory can obtain. Accreditation of the testing laboratory and validation of the assays are recommended to ensure validity of the results. Further information on the accreditation can be found from http://www.biologia.uniba.it/eca/NEWSLETTER/NS-17/Guidelines.pdf.

In conclusion, on the basis of the state of the art, conventional karyotyping can be considered a valuable and useful technique to analyze chromosomal stability during preclinical studies. If recurrent aberrations are identified, other complementary tools (e.g., CGH and/or FISH analysis) could be used to look for these aberrations because they have better sensitivity to detect a low proportion of abnormal cells.

Nonclinical aspects

Occurrence of cell abnormalities appears to be mainly related to the manufacturing process as opposed to patient-specific factors (personal communication of the experts). It is therefore important to determine during preclinical development whether the manufacturing process leads to chromosomal abnormalities.

The nonclinical discussion focused on the *in vivo* tests available for tumorigenicity testing and their reliability and relevance for the intended purposes.

Experiments performed with the use of human adipose-derived MSCs in nude mice and athymic rats with different application routes in a 3- to 6-month follow-up period were reported (personal communication of the experts). No evidence of tumor formation has been seen in such studies. MSC experts supported this observation and highlighted the fact that no evidence has been reported of tumor formation after injection of BM-derived human MSCs into immunocompromised mice. Moreover, the issue that current *in vivo* models may not be predictive for tumor formation/induction was stressed. It was highlighted that the NOD-Rag mouse model (23), which appears to be currently the most suitable model system, may not be sufficient to completely rule out the risk for tumor formation. In addition, experts mentioned that in general, the immunological status of the animals in such tumorigenicity studies should be considered. When allogeneic MSCs are used in immunocompetent mice, rejection of allogeneic cells may prevent tumor formation. Attention was drawn also to other possible model systems for tumorigenicity testing such as the Zebrafish model (24).

Clinical aspects

The majority of expert presentations at this workshop, including those from experts with experience with the use of human MSCs in clinical trials, focused on quality and preclinical findings that acknowledge the importance of these steps in the development of medicinal product in general and especially when evaluating risks in clinical application.

During the discussion on clinical aspects, the experts shared their experiences regarding the hypothetical risk for tumor development in patients receiving MSCs containing cytogenetic abnormalities. The obtained results on the use of MSCs as a cell-based medicinal product neither confirm nor exclude the risk for tumorigenicity in patients. To date, no tumors have been diagnosed in patients that would originate from administrated MSCs. Two scenarios are possible: (i) malignant transformation of the MSCs occurs *per se,* which implies both a potential risk for further malignant development of *in vitro* cultures and transformation of MSCs themselves, possibly through the immunosuppressive milieu created by these cells, and (ii) an immunosuppressive effect of MSCs, which may evoke tumor cell growth in existing malignant cells of a patient. Malignant transformation of MSCs must be further explored and monitored in a clinical setting with longer follow-up than what currently exists before any statements on potential tumorigenicity can be made.

Despite the described immunosuppressive/modulating effect of MSCs, an eventual rejection of allogeneic MSCs may be more likely to occur than for autologous cells. Also, long-lasting survival may be more likely with autologous MSCs rather than with an allogeneic product. Thus, by definition, a risk of any late-occurring adverse event would be higher for an autologous cell preparation. Considering aspects related to possible immune-toxicity and adventitious agents, the situation may be different between allogeneic and autologous cells. Allogeneic MSCs appear to be rejected in immunocompetent patients. However, even though the cells may be rejected, they may still exert therapeutic effects, for example, through paracrine effects. In addition, allogeneic MSCs may trigger an allo-immune response in immunocompetent patients, which should be taken into account when designing clinical trials for patients with autoimmune disorders, susceptible to receive a future organ transplant, or in cases in which repeated injections are planned.

Allogeneic MSCs used in the immunosuppressed transplant setting (e.g., hematopoietic stem cell

transplant) are still without conclusive results because of the small numbers of treated patients and only short-term follow-up data. The risk is evaluated to be low (>400 patients with hematopoietic stem cell transplant dosed for treatment of GvHD without demonstrated tumor formation), but no consecutive per-protocol tissue sampling has been performed. Few polymerase chain reaction–positive MSCs of donor origin (DNA) have been found in sporadic tissue sampling of patients with GvHD at an estimated level of 10^{-5} to 10^{-6} (25). It was highlighted that autologous MSCs may also be rejected in immunocompetent patients, depending on the culture process. However, the knowledge must be improved, for example, concerning patients with autoimmune diseases and use of autologous MSCs as well as concerning the tissue quality (diseased vs. healthy). Interestingly, samples for *in vitro* culturing of MSC of some donors presented the same type of cell abnormalities on separate occasions when different samples from the same donor were processed through the use of different culture methods (11). Furthermore, co-infusion of patients' autologous MSCs and allogeneic BM has been demonstrated to improve BM engraftment (autologous MSCs may support microenvironment for allogeneic BM cells). The use of MSCs from one donor for multiple recipients may create additional demands both for quality control and post-administration controls in development and manufacture.

Immunogenicity of autologous and allogeneic MSC therapy including infusion-related effects on the coagulation system is under investigation.

Experts' preliminary results suggest that MSCs may exert influence on the coagulation system. This is in line with previous reports on the inhibitory effect of MSCs on systemic intravascular coagulation in animal models (26). Thus, MSC therapy may lead to additional hemostatic complications such as thromboembolism or bleeding, which are known to be associated with cancer (27).

It was agreed that well-designed clinical trials are needed to further collect relevant clinical data. Long-term safety and efficacy trials are awaited. All experts considered the need for registries both concerning donors and patients through the use of simple questionnaires to improve user compliance. A European MSC registry already exists with an aim to collect long-term data. However, no detailed suggestions on follow-up regimens were presented.

The relevant data have been obtained from experience gathered through the use of immunosuppressive therapy in solid organ transplantation: these data revealed that the transplant population compared with the general population; that is, recipients of a kidney, liver, heart or lung transplant, have an increased risk for diverse infection-related and unrelated cancers (28). In contrast, the similarity of the pattern of increased risk of cancer in the two relevant populations (human immunodeficiency virus [HIV]/acquired immune deficiency syndrome [AIDS] and immunosuppressed transplant recipients) suggests that immune deficiency rather than other risk factors for cancer is responsible for the increased risk (29).

Risk-benefit evaluation is an inherent part of clinical practice as well as clinical trials. Additional data are needed to be collected from nonclinical animal studies. Risks of immunosuppressive effects (risk for infections, malignancies) of MSCs versus benefits demonstrated in clinical trials (anti-GvHD effect) should be further scrutinized. Risk-benefit assessment will be especially difficult in an allogeneic transplant setting, in which immunosuppressive therapy is given to all patients. Final conclusions on the tumorigenic risks of MSCs can be only achieved through the use of properly controlled and characterized MSC preparations and from long-term follow-up of treated patients.

Experience gained during the last two decades in the solid organ transplantation area revealed that although the average time to presentation of particular neoplasms occurs at distinct time intervals after transplantation, when all cancers are considered, the average latency is approximately 3 to 5 years after transplantation (30,31). Thus, the minimal time of 5 years for the long-term follow-up period is reasonable. The clinical safety program for malignancies could be based on both premarketing and postmarketing phases.

In addition, samples collected and stored at various critical steps of the cell product manufacturing process, with details about the culture conditions, could be used in evaluation of the correlation between the cell preparation and the clinical outcome. This would allow a retrospective analysis of the product, in the case of any treatment failures.

Discussion

The expert group reached agreement on several issues discussed. First, occurrence of recurrent cell abnormalities appears to be mainly related to the manufacturing process. In addition, some donor-related recurrent abnormalities have been detected. Thus, the culture conditions should be chosen to avoid a high proliferative rate (e.g., use of excessive amount of growth factors) because this may potentiate chromosomal abnormalities. Furthermore, the number of population doublings should be kept to a minimum to limit the potential for chromosomal

abnormalities. The growth rate of the cell propagation process should be justified. Pushing several samples of the cells, on an experimental basis, into senescence with the use of long-term culture was considered important to obtain further information on the potential of the culture process to lead to genetic instability under more extreme (artificial) conditions and to define a safety margin. It was also considered important to retain cell samples in the case that later testing is needed. Conventional karyotyping when combined with other techniques (CGH/SNP array, FISH) was agreed as the state of the art to evaluate putative chromosomal aberrations. All MSC processes should be evaluated for their ability to affect genetic stability of the cells. However, a karyotype or FISH analysis of each batch is only necessary in cases in which recurrent chromosomal abnormalities are found.

In current animal models, in which either human or animal cells (homologous models) are used, no evidence of tumor formation has been observed to date. However, the frequency of transformation of human MSCs is too low to detect overt tumor formation in established rodent models. The immunological status of animals may play a role in tumor development, which should be taken into account in nonclinical testing.

The risk of tumor formation of MSCs must be further explored and monitored in clinical settings with longer follow-up than what is available today before any statements on tumorigenicity can be made. The development of long-term follow-up and/or registries is encouraged to improve the knowledge on long-term effects. The time of follow-up is dependent on the specific product and impact of the treatment on the patient's health status.

In addition to tumorigenicity, further considerations should be noted such as immunogenicity of autologous and allogeneic MSC therapy, including infusion-related effects on the coagulation system, and these may also need to be considered during clinical applications. The potential risks of immunosuppressive therapy (risk for infections, malignancies) associated with the clinical use of MSCs are a central part of the benefit/risk assessment. In conclusion, an approach that is based on the totality of evidence, combining quality control with clinical observation and potentially complemented by animal studies, probably will be best suited to address existing concerns of tumorigenicity in clinical applications of MSCs.

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