Preface

The evolving paradigm, suggesting the existence of an intricate link connecting inflammatory processes with oncogenesis, finds its roots all the way back into the nineteenth century. Rudolf Virchow, one of the most prominent German physicians of his time, was the first to uncover almost 150 years ago the presence of white blood cells in tumor specimens. This observation led Virchow to suggest – largely intuitively – that carcinogenesis could occur at sites of chronic inflammation, and that a set of secreted factors produced by inflamed tissues supports neoplastic growth while helping the tumor to escape the immune system surveillance by inducing a state of so-called immunosuppression concurrently inhibiting natural elimination of malignant cells via the process currently known as apoptosis.

Today, clinical oncology data strongly support Virchow's intuition by acknowledging one out of seven newly diagnosed malignancies worldwide to result from infection and chronic inflammation. To no surprise, recognition of this astounding rate of cancer incidence caused by inflammatory processes robustly correlates with an increasing attention within both academic research environment and the biomedical industry circles towards closer evaluation of the infection–inflammation–cancer axis on a molecular level, as well as on the level of search for novel markers allowing, once targeted, to selectively restrain the oncogenic drift triggered by inflammation. The last two decades of the past millennium marked by a breathtaking evolution of molecular methods in biology – including complete sequencing of genomes in key species, nascency of proteomics and DNA microarray technologies, development of comprehensive toolkits for pathway analyses, as well as rapid maturation of chromosome engineering and gene targeting methodologies – consolidated the theoretical foundation of inflammation-associated carcinogenesis. An impressive body of evidence has been collected to develop the molecular groundwork for infection-mediated tumorigenesis with the role of reactive oxygen species, free radicals, inflammatory cytokines, such as TNFα and lymphotoxins, but also angiogenic factors secreted by an inflamed tissue to assist in its healing process, gradually becoming well recognized. Furthermore, signaling pathways known previously to primarily play either developmental or tissue homeostasis roles have now been demonstrated to critically influence the oncogenic outcome of inflammation; examples include NF-kappaB, prostaglandin/cyclooxygenase-2, and p53 pathways, the DNA repair machinery, and a family of the Toll-like receptor proteins. Intriguingly for both infection experts and oncologists, the systemic inflammation appeared to influence cancer progression during each of three stages in tumor lifetime: initiation/promotion, expansion, and invasive metastatic growth. Different mechanisms associated with the inflammation onset and its resolution have been demonstrated to play pleiotropic, yet distinct, roles at different phases of tumorigenesis.

As the number of scientific reports directly addressing the issue of inflammation-mediated tumorigenesis surpassed a notable 2,000 mark in the last year only, the value of review-type publications summarizing the findings at the cancer–inflammation boundary became almost impossible to overestimate. And yet, highest quality of the theoretical framework delivered by numerous reviews in the field provides little, if at all, room to

deduce the collinear scaffold of methodological procedures developed and validated in a variety of labs to practice the "molecular oncology of inflammation" either at the lab bench level or in the clinical diagnostics. There is a clear need to conceptualize, systematize, and standardize the existing arsenal of analytical tools developed by both oncologists and immunology experts to bring the wealth of experimental techniques under a common denominator toolkit equally valuable for biomedical researchers in academia, R&D scientists in the industry, and clinical oncologists in hospital labs.

In this light, the publication of *Inflammation and Cancer* is well timed to say the least. Although facing a challenging task of in a way shooting at a moving target because of the contemporary pace of practical arsenal development in the field, it is my sincere intention to not only collect a plethora of current methods under a single cover, but rather deliver a systematic guide to techniques addressing various aspects of experimental cancer biology selectively focusing on inflammation-mediated tumorigenesis and leaving an ample room for improvisations on a per-case basis. Apart from an unquestionable relevance of the fundamental experimental principles for a long future to come, the current collection of experimental approaches is almost certainly destined to live through the continuous waves of revisions and amendments. In my view, the significance of this book is also in setting "square zero" requirements for techniques still in the development pipeline or just added to the application pool and awaiting experimental substantiation.

The *Inflammation and Cancer* set is subdivided into four topics each consisting of chapters discussing a specific methodology with extensive citation list and reference guide for laboratory troubleshooting. Each chapter provides an introductory paragraph reviewing the relevant theoretical foundations. The following topics will be covered in the actual order as they appear in the book: *Vol. 1*, (I) Experimental Approaches to Study Chronic Inflammation-Related Carcinogenesis; (II) Oncogenic Potential of Inflammation Induced by Viral and Bacterial Infections; *Vol. 2*, (I) Crossroads of Inflammation and Cancer: Molecular Aspects; and (II) Molecular and Cellular Approaches to Diagnostics and Drug Target Discovery in Inflammation-Related Oncogenesis. It was my strong objective to maximize the page/information quality ratio of the book, but also to seek a balanced presenting of experimental procedures vs. background theoretical material.

In its present format with the scope and style of covered material, the book shall find a wide-ranging appeal among the diverse audience of scientific professionals practicing experimental oncology, immunology, cell biology, genetics, and pharmacology in both academic research and industrial R&D laboratories. Medical practitioners and clinical laboratory personnel, as well as students learning the experimental aspects of molecular medicine, will equally find helpful the roster of laboratory procedures discussed in the book. My further hope extends to a notion that the methodological arsenal discussed in its pages will in fact beget the perception of its incompleteness and stimulate further efforts in expanding the battery of experimental approaches, focusing among others on implementation of cell-based and in vivo preclinical models, to address the biology – and ultimately the therapeutic aspects – of inflammation-related tumorigenesis. On another note, fostering the rigorous scientific interactions among basic and clinical researchers aimed at further molecular demarcation of the elaborate pathways leading from inflammation to tumor formation is both the primary purpose of the book and a key metrics of its success.

Undoubtedly, this project will be next to impossible without the exceptional work of all contributing authors. It is understandably difficult to tailor – and then re-tailor again – the chapter style to reflect the editor's strategy and big-picture vision for the entire volume,

and I am very much obliged for each piece of experimental wisdom shared with the reader audience, as well as for the praiseworthy commitment of every contributing author to bear with the editor through the entire duration of the work.

On a final note, every single day we were working on this book, over 15,000 lives have been claimed worldwide due to cancer-related deaths. Current estimates give us reasons to believe that about 2,200 fatalities are actually caused by the inflammation-related oncogenesis. It is this frustrating statistic that stipulates a powerful dedication to succeed in the demanding quest of disseminating the novel diagnostic tools and therapies targeting the adverse clinical facets of inflammatory processes. My hope is that copies of these current volumes will find themselves rapidly tunneled from a library bookcase to lab benches of investigators and clinicians alike who enthusiastically seek a means to stand up against the clinical challenges reflected in the above numbers.

Volume 1

The complexity of a mechanistic basis for inflammation-associated carcinogenesis, not infrequently revolving around an intricate amalgamation of multiple biological events occurring at both cellular and molecular levels, stands as a major challenge for clinical and experimental oncology practitioners. The current advancements in deciphering the network of pathway interactions and cross-talks among different cell types at sites of inflammation or infection would be next to impossible without a battery of potent experimental tools evolved and perfected over the recent past. A synopsis of this compilation of contemporary laboratory techniques, with the emphasis placed on carcinogenic events mediated by chronic inflammation and pathogen infection, constitutes a key objective of the first out of two *Inflammation and Cancer* volumes.

Volume 1 of the book, appearing with a subtitle "Experimental Models and Practical Approaches", is composed of two parts and provides an overview of a spectrum of techniques developed to analyze the outcomes of inflammation-mediated carcinogenesis on the tissue, cellular, and molecular levels while highlighting several diagnostic aspects, such as biomarker discovery and molecular signatures evaluation. This volume as well highlights several techniques aimed at detection and analyses of pathogenic proinflammatory agents, primarily viruses and bacteria. The first part of the volume – entitled "Experimental Approaches to Study Chronic Inflammation-Related Carcinogenesis" – includes methodological chapters covering such aspects of inflammation immunology and cancer biology as a comprehensive description of surgical and molecular techniques for preparation of cancer tissue samples for molecular pattern analyses ("Collection and Preparation of Rodent Tissue Samples for Histopathological and Molecular Studies in Carcinogenesis"), description of both RNA- and protein-based bioassays to determine the cytokine expression ("Cytokine Multiplex Analysis" and "Approaches to Determine Expression of Inflammatory Cytokines"), and evaluation of chronic inflammation-associated biomarkers ("Biomarkers of Cell Proliferation in Carcinomas: Detection of Angiogenesis and Infiltrated Leukocytes" and "YKL-40: A Novel Marker Shared by Chronic Inflammation and Oncogenic Transformation"). Other chapters appearing in this part are devoted to description of bioanalytical tools that afford researchers with capabilities to evaluate the proteolytic environment of inflamed tissues ("Assessment of Local Proteolytic Milieu as a

Factor in Tumor Invasiveness and Metastasis Formation: In Vitro Collagen Degradation and Invasion Assays"), to monitor the chronic inflammation-related angiogenic events as mediators of cancer progression ("Angiogenesis Links Chronic Inflammation with Cancer"), or to study tumor-specific infiltrating immune cells via an elegant technique of their capturing and in vitro clonal expansion ("Selective Immortalization of Tumor-Specific T Cells to Establish Long-Term T-Cell Lines Maintaining Primary Cell Characteristics"). The part is concluded with a review chapter that provides an extensive and amply referenced account on experimental modeling for the most vivid example of cancer-prone inflammation process known as inflammatory bowel disease ("Inflammatory Bowel Disease: A Model of Chronic Inflammation-Induced Cancer").

The second part of Vol. 1 (entitled "Oncogenic Potential of Inflammation Induced by Viral and Bacterial Infection") consists of seven chapters that provide a compendium of experimental procedures developed to detect a panel of pathogens linked to the onset of inflammatory events that eventually lead to malignant transformation of infected organs. The list includes one of the most widely acknowledged gastrointestinal cancer-coupled bacterial pathogens *Helicobacter pylori* ("Gastric Carcinogenesis and *Helicobacter pylori* Infection" and "*Helicobacter*-Based Mouse Models of Digestive System Carcinogenesis"), and multiple viral agents such as cervical cancer-associated HPV ("Screening for Molecular Markers of Cervical Papillomavirus Infection: Overview of Methods and Their Clinical Implications" and "Detection and Genotyping Analysis of Human Papillomavirus Isolates from Liquid-Based Cervical Cytology Specimens"), common pathogen in Hodgkin's lymphomas Epstein–Barr virus ("Screening for Epstein–Barr Virus in Hodgkin's Lymphoma"), and a hepatitis C virus known to predispose infected liver cells to hepatocellular carcinoma formation ("A Hepatitis C Virus Xenograft Mouse Efficacy Model"). Remaining chapter in this part ("Gene Expression Profiling in Cervical Cancer: Identification of Novel Markers for Disease Diagnosis and Therapy") exemplifies the application of nucleic acid microarray and bioinformatics techniques to discover novel prognostic markers in HPV-associated cases of cervical cancer.

In summary, the first volume of *Inflammation and Cancer* endows cancer biologists with a collection of contemporary experimental techniques developed to assess the biochemical properties and characteristic gene expression signatures of inflamed tissues, as well as to detect and quantify inflammatory agents of viral and bacterial nature. Additional review style information on modeling the inflammation-associated carcinogenesis in experimental animals supplies a broad reference guide for the investigators intrigued by the current power of in vivo genetic tools in unveiling the molecular networks operating at the numerous anastomoses of inflammation and cancer.

Serguei V. Kozlov
Frederick, MD
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Chapter 2

Biomarkers of Cell Proliferation in Carcinomas: Detection of Angiogenesis and Infiltrated Leukocytes

Coen I.M. Baeten

Summary

Angiogenesis is an important marker for tumor growth, development, and metastasis. There are many studies to detect angiogenesis, for instance by microvessel density (MVD),though several of the studies to MVD measurement show opposite results. Measurement of MVD is a nontime-related measurement, whereas angiogenesis is a dynamic process; therefore, measurement of proliferating endothelial cells is thought to be a better method. We have shown in studies that measurement of active proliferating endothelial cells by double staining is a better marker, compared to MVD measurement. Next to angiogenesis, leukocyte infiltration in a cancer has a prognostic value. A large infiltration of leukocytes in a tumor correlates with a better survival. It is known that the correlation between leukocyte infiltration and angiogenesis is marked by adhesion molecule expression on endothelial cells. In vitro experiments show that active proliferating endothelial cells downregulate adhesion molecule expression on the cell membrane. It is generally assumed that this results in vivo in an inhibition of leukocyte infiltration in this specific area. Because immunohistochemical techniques cannot detect exact amounts of adhesion molecules in physiological environments this interaction has not been demonstrated. This chapter shows a technique based on flowcytometry by which these analyses can be performed. In short a tissue part is dissolved in a single-cell suspension, stained for specific characteristics and measured by FACS analysis. In this chapter we will show several techniques to detect proliferating endothelial cells in a tissue.

Key words: Tumor; Angiogenesis; Leukocytes; Adhesion molecule expression; Flowcytometry.

1. Introduction

Prognostic factors such as Tumor, Node, Metastasis (TNM) stage predict cancer survival. This staging shows an indication to survival, but is not sufficient to show accurate survival. Several other parameters have been proposed to improve prognostic

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criteria, among which are angiogenic potential and infiltration by inflammatory cells *(1–3)*. These two phenomena are interrelated; therefore, studies to assess the value of both phenomena for the prognosis have been made in several carcinomas. Angiogenesis is essential for tumor growth and metastasis, and is regulated by tumor cells through the production of proangiogenic factors such as vascular endothelial cell growth factors (VEGFs) and fibroblast growth factors (FGFs), and angiostatic factors such as platelet factor-4, thrombospondin-1, angiostatin, and endostatin *(4, 5)*. Detection of single or combination of these factors in tissue did not accurately show angiognic activity and survival. Markers for endothelial cells as CD31, CD34, CD105, and Von Willebrand factor show small nonsignificant differences. Different angiogenesis measurement techniques as microvessel density, image analysis software, growth-factor detection magnetic resonance imaging, and positron emission tomography are used *(6, 7)*. Measurement of angiogenesis is commonly performed by the assessment of microvessel density (MVD) *(8)*. This parameter is controversial since it might not be a reliable indicator of ongoing angiogenesis. It has been suggested that angiogenesis is best evaluated through detection of proliferating ECs *(9)*.

In contrast to angiogenesis the amount of leukocytes is assumed to be important for cancer regression, in a way that high amounts of several different leukocyte subsets show a better prognosis *(1)*. Therefore leukocyte infiltration in a carcinoma is an important prognostic factor. Though it is not known which subset exactly is important for the regression of a cancer, it is suggested that different cancers react different to several leukocyte subsets.

Communication between resident tissue cells and circulating leukocytes is mediated by the endothelial cell layer. Resident antigen-presenting cells release cytokines like TNF-α, IL-1, IFN-α, and histamine in response to an inflammatory reaction ***(10)***. Endothelial cells respond to the cytokine release by expressing adhesion molecules on their surface in order to enable leukocytes to adhere to their surface and infiltrate the surrounding area. Not only in inflammatory tissue is leukocyte infiltration important. Leukocyte infiltration in cancer tissue is thought to be a prognostic factor in a way that tumors with high leukocyte counts have a better prognosis *(11)*. Local release of proinflammatory cytokines induces biosynthesis and luminal expression of vascular adhesion molecules as ICAM-1, VCAM, and E-selectin. E-selectin is known to be of importance in the rolling of leukocytes in contrast to VCAM and ICAM which have been shown to be important in firm leukocyte endothelial cell adhesion *(12)*.

We are able to determine adhesion molecule expression on endothelial cells with our developed flowcytometric method. Using a double staining, on a single-cell suspension, with endothelial

markers and adhesion molecules we can easily detect relative expression of adhesion molecules on endothelial cells.

Next to detection of proliferating ECs, we will show in this chapter the relationship of proliferating EC with adhesion molecule expression on its membrane and the relationship with leukocyte infiltration.

2. Materials

2.1. Immunohistochemistry Needs

1. Materials:
   (a) Object slides
   (b) Cutter for 4- or 5-μm slides
   (c) Pipette
   (d) Mount slides
   (e) Microscope
   (f) Incubation room
2. Tissues: paraffin, fresh or frozen consecutive sections cut at 4 or 5 μm.
3. Solutions (*see* **Note 1**):
   (a) Deparaffinize: xylene
   (fresh from stock)
   (b) Alcohol concentrations: 100%, 96%, 70%
   (fresh from stock)
   (c) PBS 0.01 M (PBS):
   NaCl 218.8 g
   $Na_2 HPO_4 \cdot 2H_2O$ 35.4 g Dilute in 5 l distilled water
   KH_2PO_4 5.38 g
   Store at 5°C, for 2–4 months
   Needs 5× dilution before use control; for PH: 7.2–7.4 (conductivity at 25°C: 15.7 ms)
   (d) 5% BSA/PBS:
   5 g of BSA (Sigma, St Louis, MO) in 100 ml PBS
   Store at –20°C, for 6–12 months
   (e) 0.5%BSA/PBS:
   0.5 g of BSA (Sigma, St Louis, MO) in 100 ml PBS
   or
   1:10 dilution of 5%PBS/BSA in PBS.
   Store at –20°C, for 6–12 months

(f) Sodium Citrate Buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate) 2.94 g. Dilute in 1 l distilled water

Adjust pH to 6.0 with 1 N HCl

Store at 5°C, for 6–12 months

(g) DAB:

1 ml pure DAB + 9 ml Tris–HCl (0.05 M; pH 7.6) + 10 µl H_2O_2

Store at –20°C, for 6–12 months

2.2. Flowcytometry Needs

1. Materials
   (a) Flowcytometer (FACSCaliber)
   (b) Cryocutter
   (c) Warm water bath
   (d) Vortex
   (e) Centrifuge
   (f) Vacuum
   (g) Pipette
   (h) Ice
   (i) Microscope, cell-counter glass
   (j) Filter, 50 µm
   (k) FACS tubes
   (l) FACS tube holders
   (m) Eppendorf tubes
2. *Tissues.* Cut tissues, in cryo, in 30-µm sections and put them in a frozen 10-ml tube and put them on dry ice (*see* **Note 2**). When not directly used store tissue sections in a fridge at –20°C (maximum 12 months).
3. Solutions:
   (a) Collagenase:
   (b) Collagenase 5 mg/ml (50 mm of collagenase in 10 ml Bidest)
   (c) Store at –20°C, for 6–12 months
   (d) Dispase:
   (e) Dispase 1 mg/ml (10 mg Dispase in 10 ml Bidest)
   Store at –20°C, for 6–12 months
   (f) *Paraformaldehyde 0.1%.* 1% Paraformaldehyde (10 g of Paraformaldehyde diluted in 100 ml bidistilled water, pH set to 7.3 with NaOH) Store at –20°C, for 6–12 months

Before use dilute paraformaldehyde till 1% with PBS (0.01 M) 1:10

3. Methods

For the immunohistochemical methods paraffin, fresh as well as fresh frozen tissues, can be used. The protocol we show is made for paraffin-embedded tissues. When fresh or frozen tissues are used use an extra step of incubation with 1% paraformaldehyde (follow protocol flowcytometry) is needed instead of the deparaffinization steps. This protocol can be followed afterward.

For all fresh frozen tissues, best results are obtained when the tissues are directly fixated when obtained; proliferation of cells is dependent on nutrients. Next to proliferation the amount of adhesion molecule expression will also decrease in time if the tissue is not fixated (*see* **Note 2**).

The following protocols will guide you through a number of techniques to detect proliferating EC, leukocytes, and adhesion molecule expression on EC.

3.1. Ongoing Angiogenesis Measured by Immunohistochemistry

The classical way to detect angiogenesis by MVD detection has been shown to register a static situation of the number of vessels in a tissue. We and others have shown that the number of active proliferating EC is a better method to detect ongoing angiogenesis. This protocol shows how a double staining of Ki67 (proliferation marker) with EC marker cocktail (CD31/CD34) can be performed.

3.1.1. Protocol: Ongoing Angiogenesis Measured by Immunohistochemistry

1. For this research use adjacent sections, because after staining the tumor sites might be difficult to find, it is useful to stain one of the adjacent sections with hematoxilin/eosin. This gives a better result than staining the section with hematoxilin/eosin, because the blue staining might be difficult to see after counterstaining with hematoxilin.
2. Deparaffinize (2× xylene for 5 min)
3. Rehydrate (2× 100% alcohol; 1× Alcohol 96%; 1× alcohol 70%; (each 5 min)
4. Twenty minutes in methanol + 3% H_2O_2 (to eliminate endogen peroxidase)
5. Rinse with H_2O
6. Citrate-buffer pH 6; incubate at boiling temperature for 10 min. Permeabilization of the cell membrane is important for Ki-67 staining in the nucleus.
7. Thirty minutes cooling down
8. 3 × wash with PBS
9. Dry slides
10. Five percent BSA in PBS; 100 µl per slide, use a humid environment for 30 min. Blocks a nonspecific binding

11. Clean slides by putting them in an oblique position
12. Add First antibody Ki-67 (Rabbit anti-Human; NeoMarker, Freemont, CA.) 100 µl per slide, incubate for 60 min in a humid environment.

    1:200 dilution ⇒ 10 µl in 2 ml 0.5% BSA in PBS (*see* **Notes 3** and **4**)
13. 3× wash with PBS
14. Add complementary α-rabbit[b] (Swine-anti-rabbit[biotinc]; DAKO, Glostrup, Denmark),

    100 µl per slide, incubate for 30 min in a humid environment

    1:200 dilution ⇒ 5 µl + 1 ml 0.5% BSA in PBS 0.5
15. 3× wash with PBS
16. Avidine-Bovine-Complex HRP (ABC-complex HRP; DAKO, Glostrup, Denmark), firm protocol, 100 µl per slide, incubate for 30 min in a humid environment
17. 3 × wash with PBS
18. DAB solution (DAB, Sigma, Zwijndrecht, the Netherlands)

    ⇒ *Work with gloves (DAB is carcinogenic)*

    1 ml pure DAB + 9 ml Tris–HCl (0.05 M; pH 7.6) + 10 µl H_2O_2: DAB is photosensitive, use dark environment, use 100 µl per slide, control staining under microscope, when clear specific staining appears stop the reaction by adding H_2O
19. 2× wash with PBS
20. Antibody cocktail CD31 + CD34 (both mouse-antihuman; DAKO(CD31)/QBEND-10, novocastra, Uden, The Netherlands(CD34)), 100 µl per slide, incubate for 60 min in a humid environment

    1:100/1:50 dilution (CD31/CD34) 10 µl + 20 µl + 1 ml
21. 3× wash with PBS
22. Add complementary α-mouse[b] (anti-rabbit[biotinc]), 100 µl per slide, incubate for 30 min in a humid environment

    1:200 dilution ⇒ 5µl + 1 ml 0.5% BSA in PBS 0.5
23. 3× wash with PBS
24. Avidine-Bovine-Complex AP (ABC-complex AP; DAKO), company's protocol, 100 µl per slide, incubate for 30 min in a humid environment
25. 3 × wash with PBS
26. Alkaline phosphatase substrate KIT III Blue (Vector Laboratories, Inc., Burlingame, CA); photosensitive, use dark environment, use 100 µl per slide, control staining under

microscope, when nice specific staining stop the reaction by adding H_2O use Tris 0.8 M pH 8.2 dilute the alkalic phosphatase (AP) in

27. Add a film of imsol mount to protect AP from alcohol
28. Let slides air dry completely
29. Dehydrate and mount slides

3.2. Immune System (Leukocyte Subsets)

The presence of immune cells in tumors is generally seen as a favorable prognostic parameter. This is evident for leukocyte subsets, including T-lymphocytes, PMNs, and macrophages ***(13, 14)***. However, for tumor-associated macrophages adverse effects have been described ***(15, 16)***. Tumor infiltration by leukocytes is regulated by a number of inflammatory cytokines, the combination of which presumably determines the amount and composition of the infiltrate. In addition, infiltration is also affected by regulators of angiogenesis. Both inhibitory and stimulatory effects were found for VEGF and other angiogenic growth factors during angiogenesis ***(17–19)***. Angiogenesis is inversely correlated with leukocyte infiltration in a way that leukocyte infiltration is lower when high angiogenic activity is measured in a tissue (also cancer tissue). Therefore leukocyte infiltration is indicative of endothelial cell proliferation level.

3.2.1. Protocol: Immunohistochemical Staining of the Immune System (Leukocyte Subsets)

1. Deparaffin (2× xylene for 5 min)
2. Rehydrate (2× 100% alcohol; 1× Alcohol 96%; 1× alcohol 70%; (each 5 min))
3. Twenty minutes in methanol + 3% H_2O_2 (to eliminate endogen peroxidase)
4. Rinse with H_2O
5. Citrate buffer pH 6; incubate at boiling temperature for 10 min
6. Thirty minutes cooling down
7. 3× wash with PBS
8. Dry slides
9. Five percent BSA/PBS 100 µl per slide, use a humid environment for 30 min
10. Clean slides by putting them in an oblique position
11. Add First antibody (all mouse-antihuman):

CD3 (DAKO)	1:50 dilution
CD8 (Novocastra, Valkenswaard, the Netherlands)	1:100 dilution
CD16 (NeoMarker, Freemont, CA.)	1:50 dilution
CD20 (DAKO)	1:1000 dilution
CD68 (DAKO)	1:100 dilution

Hundred microliters per slide, incubate for 60 min in a humid environment.
All dilutions are made with 0.5% BSA in PBS

12. 3 × wash with PBS
13. Add complementary α-mouse[b] IG (rabbit-antimouse[biotine]; DAKO, Glostrup, Denmark), 100 µl per slide, incubate for 30 min in a humid environment

    1:200 dilution ⇒ 5 µl + 1 ml 0.5% BSA in PBS 0.5
14. 3× wash with PBS
15. Avidine-Bovine-Complex HRP (ABC-complex HRP; DAKO, Glostrup, Denmark), firm protocol, 100 µl per slide, incubate for 30 min in a humid environment
16. 3 × wash with PBS
17. DAB-solution (DAB, Sigma, Zwijndrecht, the Netherlands)

    ⇒ *work with gloves (DAB is carcinogenic)*

    1 ml pure DAB + 9 ml Tris–HCl (0.05 M; pH 7.6) + 10 µl H_2O_2: DAB is photosensitive, use dark environment, use 100 µl per slide, control staining under microscope, when nice specific staining stop the reaction by adding H_2O
18. 2 × wash with PBS
19. Counterstain with hematoxilin
20. Dehydrate (1× 70% ethanol, 1× 96% ethanol, 2× 100%ethanol (each for 5 min))
21. Mount slides

3.3. Adhesion Molecule Expression on EC

Detection of amount or percentage of endothelial cells (EC) (not blood vessels) and adhesion molecules on EC is not possible with immunohistochemical methods. It is plausible that each EC has the capacity to express adhesion molecules on its surface. Therefore single-cell suspensions are needed to detect the percentage of EC in a tissue. We developed a technique based on flowcytometric detection of endothelial cells in a tissue solution. With this technique an accurate percentage of endothelial cells in a tissue can be detected. For this technique paraffin-embedded, fresh frozen as well as fresh tissue can be used. Thirty-micrometer tissue sections are initially dissolved to form a single-cell suspension. This suspension can be stained for any set of specific markers; therefore, this technique is not solely limited to endothelial cell detection, but is a general method to detect leukocytes, tumor cells, etc. This technique has a very high reproducibility no interobserver variation, and is easy to perform. For this protocol we used frozen tissues, because a number of adhesion molecules are not detectable after paraffin embedding. When paraffin-embedded tissues are used in other

setting use **steps 1** and **2** shown in immunohistochemical protocol instead of paraformaldehyde fixation.

The protocol shows three sections **(Subheadings 3.3.1–3.3.3)** to guide a reader through the entire process.

3.3.1. Protocol: Standard Preparation of Single Cells for Flowcytometric Analysis

Safety: Be careful with tumor samples and even with stained cells!

1. The tissue sections of 30 µm are cut in a cryocutter. The tissues can be put directly in a 10-ml tube; if not used immediately, the tissues can be stored at –20°C.
2. All materials and solutions need to be prepared before starting the protocol.
3. Tissues in a 10-ml tube need fixation, a 1/2 or 1 ml paraformaldehyde 1% is added and incubated for 60 min
4. After 1 h use centrifuge (RZB = 689) for 5 min.

   Then take the fluid away using the vacuum
5. Single-cell suspension preparation:

   Collagenase 5 mg/ml + Dispase 1 mg/ml (ten collagenase: one dispase) (*see* **Notes 5** and **6**)

   Add 1 ml collagenase/dispase solution to every tube

   Put tubes for 15 min in a water bath 37°C

   Use a 1,000-µl pipette to triturate the liquid with tissue parts.

   Repetitive pipetting will result in a single-cell suspension.

   Make a single-cell suspension (if this does not work out for the tissue a brief titration in time and concentration of collagenase and dispase must be performed)
6. After 15 min, put the tubes directly on ice and use 9 ml PBS to fill the tube (this stops the enzyme reaction)
7. Take cell counter and control the single-cell suspension (all single cells)
8. Use centrifuge (RZB = 398) for 5 min.

   Remove the fluid using the vacuum, be careful not to lose cells in each vacuum step!! Repeat the rinsing step twice
9. Divide the single-cell suspension over FACS tubes:

   For each 10-ml tube (after vacuum):

   Add *X* times 200 µl 0.5% BSA in PBS, where

   X is total number of FACS tubes for one tissue sample needed for the experiment

   Suspend the fluid using the vortex and divide it in the X FACS tubes
10. Use centrifuge (RZB = 398) for 5 min.

Then take the fluid away using the vacuum.
From this step on perform all experiments on ice!!!

3.3.2. Protocol: Detection of Endothelial Cells in Single-Cell Suspension

1. For every step a control sample must be used.
2. Make primary antigen solution ⇒ CD-31 (mouse-antihuman; DAKO)

   1:100 dilution in 0.5% BSA in PBS. Use 20 µl solution per FACS tube. Shake tubes (vortex) to suspense the single cells in the solution. Incubate for 1 h (on ice)
3. After 1 h add 200 µl, 0.5% BSA in PBS per FACS tube

   Use centrifuge RZB = 398 for 5 min.

   Then take the fluid away using the vacuum.

   Repeat rinsing two more times
4. Make secondary antigen solution ⇒ biotin-conjugated rabbit-antimouse (DAKO)

   1:50 dilution in 0.5% BSA in PBS

   20 µl solution/FACS-tube. Shake tubes (vortex) to suspend the single cells in the solution

   Incubate for 1 h (on ice)
5. After 1 h add 200 µl 0.5% BSA in PBS per FACS tube

   Use centrifuge RZB = 398 for 5 min.

   Then take the fluid away using the vacuum.

   Repeat rinsing two more times
6. Make tertiary antigen solution ⇒ phycoerythrin (PE)-conjugated streptavidin (10 µg/ml; DAKO) 1:25 dilution in 0.5% BSA in PBS

   Twenty microliters solution/FACS tube. Shake tubes (vortex) to suspend the single cells in the solution

   Let antigens incubate for 1/2 h (on ice and cover because coloring is light sensitive)
7. After 1 h put 200 µl 0.5% BSA in PBS/FACS tube

   Use centrifuge RZB = 398 for 5 min

   Then take the fluid away using the vacuum

   Repeat rinsing two more times
8. The cells are now ready for FACS analysis

3.3.3. Protocol: Adhesion Molecule Expression on Endothelial Cells

For double staining of adhesion molecules on the surface of endothelial cells, **step 18** should be skipped and the following steps need to be performed (*see* **Notes 7–9**):

1. Direct FITC-conjugated antibodies are needed

   ICAM-1 1:50 dilution in 0.5% BSA in PBS + 5% normal mouse serum

VCAM-1 1:50 dilution in 0.5% BSA in PBS + 5% normal mouse serum

E-selectin 1:40 dilution in 0.5% BSA in PBS + 5% normal mouse serum

The presence of 5% normal mouse serum is used to prevent aspecific binding to the primary staining.

20 µ solution/FACS tube. Shake tubes (vortex) to suspense the single cells in the solution

Let antigens incubate for 2 h (on ice and cover because coloring is light sensitive)

2. After 2 h put 200 µl 0.5% BSA in PBS/FACS tube

   Use centrifuge RZB = 398 for 5 min.

   Then take the fluid away using the vacuum.

   Repeat rinsing two more times

3. The cells are now ready for FACS analysis (**Fig. 1**) (*see* **Note 10**).

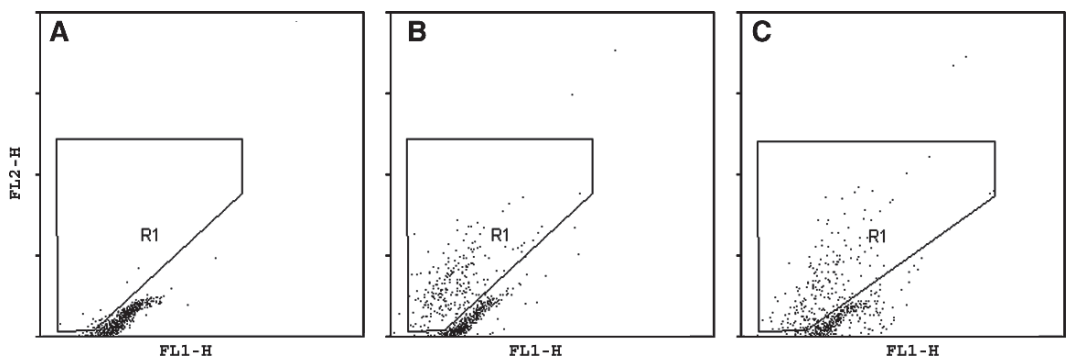

Fig. 1. Example of flowcytometric results. (A) is a control. The gate plotted shows only 0.4% of hits. In the CD31-stained solution (B) this number of hits (CD31 positive) is 11.35%. This means 11% of the total amount of cells in this tissue (colorectal cancer) is endothelial cell. Furthermore, the *x*-value in the plotted gate is 6.5; this means that the standard autoimmunefluorescence, for FL1-H, of the endothelial cells is 6.5. This value of 6.5 can be set to zero when compensation with software delivered with a flowcytometer is used. In (C) the amount of adhesion molecule ICAM-1 on endothelial cells is plotted. What can be seen is that all positive ICAM-1 cells move to the right, not only endothelial cells. The x-value in the gate is 10.3; this is the parameter for the amount of ICAM-1 positivity of the endothelial cells. There is an equal amount of cells stained within the gate (11.30%, all endothelial cells) which means no loss of endothelial cells in the negative cloud right of the gate. In some cases this cloud fully disappears in the endothelial negative cloud; therefore, compensation with software can be used.

4. Notes

1. All solutions are prepared with distilled water, unless stated otherwise.
2. Consecutive tissues are essential in immunohistochemistry to identify tumor regions in HE-stained tissues and be marked on the double-stained (Ki67-CD31/34) slides.
3. Antibodies from different stocks or different commercial sources might need other dilution.
4. All antibodies collagenase and dispase dilutions can be prepared, aliquoted, and stored in a freezer at –20°C to ensure consistent conditions.
5. Repeated vigorous pipetting with a 1-ml pipette during collagenase and dispase treatment results in a single-cell suspension. The use of a mesh is also possible, though some tissue can get retained by the mesh and this might influence the results.
6. Tissues should be handled carefully particularly in the fresh frozen tissues. When cells are damaged or too many cell clots still exist, the FACS analysis will not accurately show percentages. Different tissues might need different concentration of collagenase/dispase solution and different incubation time of collagenase/dispase at 37°C waterbath. A microscopic evaluation of the single-cell conditions can be performed after preparation of the single-cell suspension. Propidium iodide (20 µg/ml in PBS, permeabilization in 70% ethanol) can be used as a control for intact cells.
7. Before flow-cytometric measurement the cells need to be suspended with a vortex resulting in an adequate single-cell suspension.
8. Be aware that the fluorescent particles are light sensitive; therefore, covering of the samples, after admitting, is necessary.
9. Quantification with flow cytometry (in our study a FACSCaliber), with at least two detection lasers (FITC (525 nm) and APC (660 nm)) was used. The use of compensation rate for red or green light can be helpful.
10. This technique can be adapted for many strategies to identify different cell types in a tissue. The basic procedure, preparation of a single-cell suspension, can be used in almost all tissues.

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